

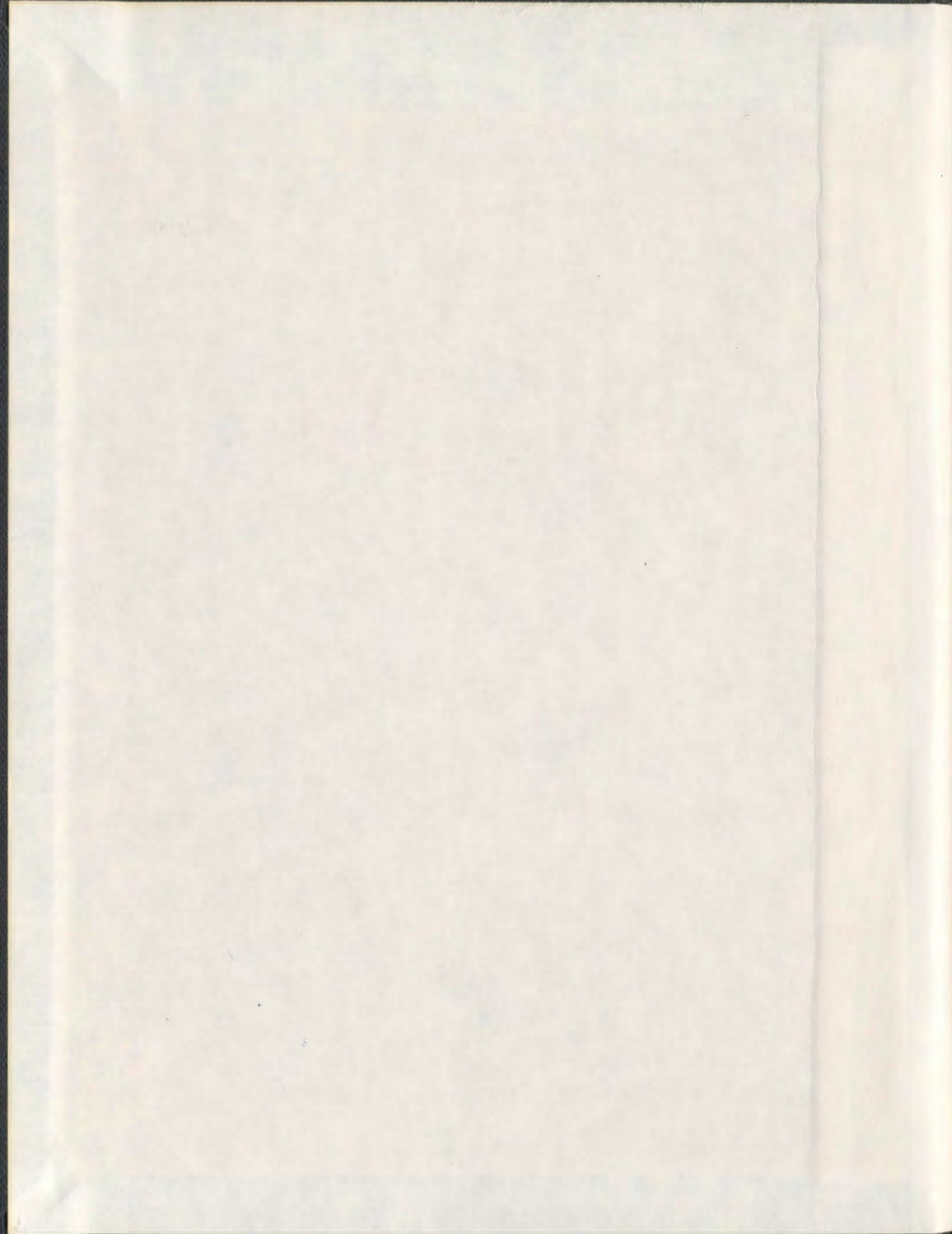
**CD8⁺ CYTOTOXIC T LYMPHOCYTES IN HUMAN
IMMUNODEFICIENCY VIRUS INFECTION**

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CD8⁺ Cytotoxic T Lymphocytes in Human Immunodeficiency Virus Infection

by

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A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements of

the degree of Doctor of Philosophy

Basic Science Division, Faculty of Medicine

Memorial University of Newfoundland

April 2003



St. John's

Newfoundland

ABSTRACT

Cytotoxic T lymphocytes (CTL) are important mediators of cellular immune responses and play a central protective role in many human viral infections. In human immunodeficiency virus (HIV) infection a vigorous antiviral CTL response is induced. However, defining the precise role of HIV-specific CTL over the clinical course of HIV infection has been the subject of much speculation. Because anti-HIV CTL kill infected CD4⁺ and antigen-presenting cells, some investigators suggest that they mediate CD4⁺ T cell depletion, contributing to the immunopathology associated with progression to AIDS. An extensive body of evidence now supports at least a partially protective role for HIV-specific CTL in controlling viral replication and delaying disease progression. However it is also clear that anti-HIV CTL fail to control HIV infection indefinitely. Multiple diverse mechanisms with the potential for contributing to the progressive deterioration of HIV-specific CTL have been shown to arise over the natural course of HIV infection, but the relative contribution of the various mechanisms to CD8⁺ T cell dysfunction has not yet been clearly defined.

The present study examined properties of anti-HIV CTL at various stages of disease. The primary objectives of the experiments were to further elucidate the biological relevance of HIV-specific CTL, and identify reasons for their inadequate immunoprotective role. The CD8⁺ T cell population that persists through the advanced stages of infection was examined, to determine if characteristics peculiar to progressive infection were imposed on CTL over the course of disease. HIV-specific memory T cell responses of individuals

at various stages of infection were examined longitudinally, to identify immunological and virological parameters affecting the maintenance of HIV-specific memory T cells, and to determine the relationship between the expanded CD8⁺CD28⁻ T cell population and disease progression.

The studies clearly demonstrate that CD8⁺ T cells retain many functional and genetic attributes through the advanced stages of HIV infection. However, an *in vivo* defect in the ability to generate an effector CTL response becomes evident in the later stages of disease, despite the persistence of intact HIV-specific memory CTL precursors. This defect is overcome with effective therapy, allowing reconstitution of CD8⁺ T cell immune responses, including HIV-specific CTL activity, even following prolonged periods of advanced infection. The accumulation of CD8⁺CD28⁻ HIV-specific CTL was found to be associated with progressive disease, and these cells showed a skewed T cell receptor (TCR) beta chain variable gene (βV) usage and decreased cytokine output in comparison to their CD8⁺CD28⁺ counterparts. Most HIV-infected individuals maintained stable anti-HIV memory T cells over prolonged periods of effective viral suppression, but a minority demonstrated a specific defect in memory T cell maintenance that was associated with present or past CD4⁺ T cell depletion.

The results of this thesis confirm the importance of HIV-specific CTL in controlling HIV disease progression and identify some of the determinants related to the CD8⁺ T cell dysfunction that is observed concurrent with progression to AIDS.

ACKNOWLEDGEMENTS

The work presented in this thesis was carried out in the research laboratory of Dr. Michael Grant, Faculty of Medicine, Memorial University of Newfoundland. I would like to extend my sincere appreciation to Dr. Grant for allowing me to undertake this project under his supervision. Dr. Grant is a wonderful mentor with an extraordinary ability to comprehend and explain complex concepts. His willingness to listen and discuss ideas, even those bordering on the 'bizarre', is a rare and much appreciated trait. I thank him for his guidance, encouragement, and advice. Above all else, however, I thank him for his patience and understanding, which were required (and given) in more than copious amounts during certain phases of this project!

I am grateful to the other members of my supervisory committee, Dr. Andrejs Liepins and Dr. David Haegert, who were always available to discuss strategies and offer constructive criticism.

I wish to acknowledge the support and technical assistance I received from co-workers and fellow graduate students, most notably Maureen Gallant, Ingrid Pardoe, Rodney Russell, Lisa Barrett, Rosemarie Mason, Sharon Oldford, and David Spurrell. Thanks guys – I couldn't have done it without you. A special thank-you to Lisa for handling the painful, last minute 'bits and pieces'.

I extend heartfelt thanks to my long time Immunology buddies, Dr. Sheila Drover, Dianne Codner, and Mary Primmer, for their never-ending support and encouragement. Friends like you are hard to come by.

I would also like to acknowledge Dr. William Marshall, former Professor of Immunology. Dr. Marshall was my employer for many years, as well as my MSc supervisor. I feel privileged to have worked and studied under his guidance for so many years. His enthusiasm for science, combined with his incredible wisdom, has inspired me in numerous ways, and I therefore dedicate this thesis to him.

I am indebted to the Canadian Institutes of Health Research (CIHR), the Canadian Foundation for AIDS Research (CANFAR), and the School of Graduate Studies, Memorial University of Newfoundland for providing financial support for this research project.

Finally I wish to thank my family; my husband Ted, and my two sons Michael and Timothy. They have supported me unconditionally throughout this project and for that I am extremely grateful. My accomplishment is your accomplishment. I love you madly, and thank you from the bottom of my heart.

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LIST OF ABBREVIATIONS

ACD	Acid-citrate dextrose
AIDS	Acquired immunodeficiency syndrome
AMLR	Autologous mixed lymphocyte reaction
APC	Antigen presenting cell
APL	Altered peptide ligand
ATTC	American type culture collection
BLCL	B lymphoblastoid cell line
βV^*	Beta chain variable gene
BSA	Bovine serum albumin
CD	Cluster of differentiation
^{51}Cr	Chromium 51
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
$C\alpha$	Alpha chain constant gene
E:T	Effector to target ratio
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetic acid
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HTLV	Human T lymphotropic virus
IEL	Intraepithelial lymphocyte
IFN- γ	Interferon gamma
IL-2	Interleukin-2
KIR	Killer inhibitory receptor
LCMV	Lymphocytic choriomeningitis virus
LTNP	Long term non-progressor
MHC	Major histocompatibility complex
NK	Natural killer
NNRTI	Non-nucleoside analogue reverse transcriptase inhibitor
NRTI	Nucleoside analogue reverse transcriptase inhibitor
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohemagglutinin
PI	Protease inhibitor

PMA	Phorbol 12-myristate 13-acetate
RT	Reverse transcriptase
SD	Standard deviation
SIV	Simian immunodeficiency virus
TCR	T cell receptor

* The TCR gene nomenclature used throughout this thesis is somewhat different from the current official nomenclature. The international immunogenetics information system (IMGT) nomenclature (website found at <http://imgt.cines.fr>) has been officially recognized as the scientific standard (Wain HM et al, Guidelines for Human Gene Nomenclature, *Immunity* 2002; 79:464-470). However, chapter 2 of this thesis was published prior to this decision, utilizing the older TCR nomenclature. It was therefore decided to continue using the older nomenclature throughout the thesis, for the sake of consistency. An example of the difference is given below:

For 'T cell receptor beta chain variable gene 12'

Nomenclature used in this thesis: TCR β V12

Official IMGT nomenclature: TRBV12

Preface and Co-authorship Statement

In accordance with the thesis guidelines from the School of Graduate Studies, Memorial University of Newfoundland, this Ph.D. thesis is written in manuscript format. A literature review is presented in chapter 1, followed by a description of the thesis proposal and the specific research objectives. Chapters 2-5 are 'stand alone' research papers describing the results of the study. Chapter 6 is an integrated discussion. The literature review presented in chapter 1 and the results described in chapters 2-5 appear in the following peer-reviewed publications:

1. **Gamberg J**, Barrett L, Bowmer M, Howley C, Grant M. Immune reconstitution and viral stimulation are required to restore HIV-specific CD8 cell responses following advanced infection. *Journal of Clinical Immunology*, in press.
2. **Gamberg J**, Pardoe I, Bowmer M, Howley C, Grant M. Lack of CD28 expression on HIV-specific cytotoxic T lymphocytes is associated with disease progression. *Immunology and Cell Biology*, in press.
3. **Gamberg J**, Barrett L, Bowmer M, Howley C, Grant M. Factors related to loss of HIV-specific cytotoxic T lymphocyte activity. *AIDS*, in press.
4. **Gamberg J**, Grant M. Cytotoxic T lymphocytes in human immunodeficiency virus type-1 infection: important or impotent? *Clinical and Applied Immunology Reviews* 2000; 1:17-36.

5. **Gamberg JC**, Bowmer MI, Trahey JC, Campbell CM, Pardoe I, Grant MD.

Functional and genetic integrity of the CD8 T-cell repertoire in advanced HIV infection. *AIDS* 1999; 13:2043-2053.

The candidate was responsible for the design of the experiments, the data analysis, the practical aspects of the research, and manuscript preparation, with the following exceptions. Ingrid Pardoe conducted the experiments examining TCR β V diversity, and Lisa Barrett performed some of the CD4⁺ T cell proliferation assays and provided assistance with the statistical analysis.

The candidate was also involved in collaborations with other researchers in the laboratory which resulted in the following publications:

1. Kottlil S, **Gamberg J**, Bowmer I, Trahey J, Howley C, Gallant M, Grant M. Human immunodeficiency virus type 1 replication, immune activation, and circulating cytotoxic T cells against uninfected CD4⁺ T cells. *J Clin Immunol* 2000; 20:175-186.
2. Kottlil S, Bowmer M, Trahey J, Howley C, **Gamberg J**, Grant M. Fas/FasL-independent activation-induced cell death of T lymphocytes from HIV-infected individuals occurs without DNA fragmentation. *Cell Immunol* 2001; 25:1-11.

CHAPTER 1

Cytotoxic T Lymphocytes in Human Immunodeficiency Virus Type-1 Infection: Important or Impotent?

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Abbreviations: HIV, human immunodeficiency virus; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; LCMV, lymphocytic choriomeningitis virus; HLA, human leukocyte antigen; SIV, simian immunodeficiency virus; TCR, T cell receptor; KIR, killer inhibitory receptor; LTNP, long-term non-progressor; APL, altered peptide ligand; HAART, highly active antiretroviral therapy.

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1.1 Abstract

Virus-specific cytotoxic T lymphocytes (CTL) potentially protect against viral infection, or once infection has occurred, limit viral replication and prevent the development of associated disease. Although there is evidence that generation of anti-HIV CTL allows a small minority of individuals to resist HIV infection, once HIV infection does occur, even potent CTL responses fail to control HIV replication sufficiently for protection from disease progression. Despite this ultimate failure, many investigators believe that an effective anti-HIV CTL response at least temporarily limits HIV replication and delays disease progression. In this review, we describe the salient characteristics of anti-HIV CTL, discuss some of the evidence that anti-HIV CTL modulate viral replication and disease course and present several of the more prominent explanations for their eventual failure. We conclude that strategies for modulating the CTL response in HIV-infected individuals should be applied together with highly active antiretroviral therapy to maintain or elicit anti-HIV CTL responses. If such strategies reduce dependence on antiretroviral therapy for suppression of HIV replication, they will provide direct evidence that anti-HIV CTL influence the rate of HIV replication and disease progression.

Key words: Cytotoxic T lymphocytes, HIV, AIDS

1.2 Introduction

Through their specific lysis of infected host cells, cytotoxic T lymphocytes (CTL) are important mediators of protective cellular immunity. CTL are primarily $CD8^+$ T lymphocytes that recognize foreign peptides presented by class I major histocompatibility complex (MHC) molecules [1]. When the antigen specific T cell receptor (TCR) of $CD8^+$ T cells encounters antigenic peptide/MHC complexes and receives appropriate co-stimulatory signals, the T cell becomes activated, divides and differentiates into a CTL. Subsequent contact with cells expressing that antigen triggers CTL-mediated killing. CTL normally kill their target cells either by releasing perforin, which polymerizes and forms pores on the target cell membrane, or by expressing fas ligand at their cell surface. Fas ligand triggers programmed cell death by binding the fas receptor on sensitive target cells [2].

Studies of lymphocytic choriomeningitis virus (LCMV) infection in mice first demonstrated the central role of CTL in viral infections and also introduced immunologists to their ambivalent potency [1,3]. LCMV is not cytopathic, therefore, it is quite possible for virus and host to coexist. Under certain conditions, LCMV-specific CTL effectively control viral replication, however, when CTL arise too late against an already entrenched virus, they can mediate significant, and even lethal immunopathology. Anti-viral CTL have subsequently been shown to play an important protective role in controlling many human viral infections such as influenza, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) [4-6]. However, it has been proposed that HIV-specific CTL

contribute to the immunodeficiency associated with progressive HIV infection by eliminating HIV-infected CD4⁺ T cells and antigen presenting cells [7].

1.3 CTL Responses to HIV infection

Infection with HIV induces a vigorous antiviral cellular immune response. HIV-specific CTL were first described in 1987 with the direct detection of CTL specific for both gag and env gene products in freshly-isolated peripheral blood mononuclear cells (PBMC) [8]. This level of CTL activity was hitherto unreported for viral infections and subsequently has only otherwise been described for chronic infections with human T lymphotropic virus type 1 (HTLV-1), another T lymphotropic retrovirus [9].

The anti-HIV CTL response is as remarkable for its diversity as for its magnitude. Most infected individuals harbor CTL specific for multiple HIV peptide epitopes presented by several HLA molecules [10,11]. Anti-HIV CTL responses involving six or more different peptides from an assortment of HIV proteins are common and it is very likely that not all responses are detected [12]. At the opposite extreme, cases where the anti-HIV CTL response was apparently directed against only a single gag epitope for several years have also been described [13].

Although anti-HIV CTL are predominantly directed against peptide products of the major gag, pol and env genes, the regulatory protein products of the tat and nef genes are also targeted [14]. A large number of HIV CTL peptide epitopes have been identified and recorded in the Los Alamos HIV Molecular Immunology database [15]. No particular pattern of epitope distribution is apparent as epitopes cluster in some regions of

the viral genome and are evenly distributed through other regions. In many instances, multiple HLA class I molecules can present the same epitope and although individuals generally respond to HIV in a predictable manner based on their HLA type, there are numerous examples of individuals with a common class I molecule not responding to the same epitope [14,16-18]. These studies indicate that multiple complex factors govern the potentially extensive CTL response against HIV.

1.4 Characteristics of anti-HIV CTL

Perhaps the most notable characteristic of anti-HIV CTL is their extremely high frequency in peripheral blood. This high CTL frequency relates to chronic HIV replication and the attendant stimulation of CD8⁺ T cells. Persistent HIV replication and immune activation appears to affect the phenotype of both anti-HIV CTL and an inordinately large proportion of bystander CD8⁺ T cells. The percentage of CD8⁺ T cells expressing activation markers such as CD38 and HLA-DR is significantly elevated in progressive HIV infection, and inversely correlated with the percentage of CD8⁺ T cells expressing CD28 [19,20]. Circulating anti-HIV CTL activity is concentrated within the CD38⁺HLA-DR⁺CD28⁻ population, which decreases in size in parallel with decreased viral replication [20,21]. This supports the general view that CD8⁺CD28⁻ T cells are effector cells turning over rapidly in vivo from CD28⁺ precursors. Loss of CD28 expression in vitro signifies replicative senescence and the shortened telomeres of CD28⁻ cells circulating in HIV infection may indicate the antigen-driven accumulation of HIV-specific CTL no longer capable of clonal expansion [22]. However, recent data showing

that some anti-HIV CTL memory precursor cells are CD28⁻ and that certain CTL specificities may be confined to a CD28⁻ population suggest this view of the origin of CD28⁻CD8⁺ T cells in HIV infection is at least partly an oversimplification [23-25]. Mobilization of inherently CD28⁻CD8⁺ T cells from mucosal sites of HIV replication, such as the gut, has also been proposed to explain the rapid and pronounced increase in circulating CD28⁻CD8⁺ T cells in HIV infection [26]. Intestinal intraepithelial lymphocytes (IEL) have an extremely limited TCR repertoire, yet IEL from simian immunodeficiency virus (SIV)-infected macaques can mediate CTL activity against several SIV antigens [27-29]. Along with their specificity and oligoclonality, other intriguing similarities between IEL and the circulating CD28⁻CD8⁺ T cells in HIV infection include constitutive cytotoxicity, autoreactivity and poor proliferative potential [30,31]. However, there is no direct evidence that IEL migrate to the peripheral circulation.

Chronic activation of anti-HIV CTL probably underlies several other phenotypic peculiarities that develop in progressive HIV infection. Under some conditions, receptors first described on natural killer (NK) cells and termed killer inhibitory receptors (KIR), are also expressed on T cells. Engagement of self-MHC class I molecules by these receptors suppresses activation of the cytotoxic cell's cytolytic machinery. For NK cells, this appropriately allows expression of cytotoxicity against allogeneic cells or tumor cells that have down-regulated expression of class I MHC molecules, while preventing the killing of cells expressing self MHC class I molecules. Increased expression of KIR on T cells in HIV-infected individuals reduces anti-HIV CTL-mediated lysis of autologous target cells in vitro [32,33]. There is speculation that KIR expression on chronically

activated T cells reflects an adaptive mechanism reducing immunopathology associated with unresolved CTL activation, but expression of these receptors could also reduce the efficiency of CD8⁺ T cell-mediated viral suppression.

Reduced expression of components of the TCR signal transducing complex also compromises the effector function of T cells from HIV-infected individuals [34,35]. Overnight culture of peripheral blood T cells from HIV-infected individuals with interleukin-2 (IL-2) restores expression of the CD3 zeta chain to normal levels and rescues previously undetectable HIV-specific CTL activity [35]. Unfortunately, the effect of in vivo IL-2 administration on HIV-specific CTL activity has not yet been reported. The mechanism behind down-regulation of components of the TCR signal transducing complex remains obscure, but similar findings have been reported in cancer patients with high tumor burdens [36].

1.5 Evidence of a protective role for anti-HIV CTL

Untreated HIV infection usually progresses to AIDS despite a vigorous CTL response. This raises speculation as to the precise role of CTL over the natural course of HIV infection and, indeed, some studies even suggest that anti-HIV CTL have no effect towards reducing the risk of death in HIV-infected individuals [37]. In a recent study, when CD8⁺ T cells were depleted from macaques prior to SIV infection, virus replication reached higher levels in primary infection [38]. Similarly, when CD8⁺ T cells were depleted from chronically-infected macaques, a rapid and marked increase in viremia occurred that was again suppressed coincident with the reappearance of SIV-specific

CD8⁺ T cells [38,39]. These studies provide direct evidence of a role for cell-mediated immune responses in limiting HIV replication. An extensive body of evidence now supports at least a partially protective role for anti-HIV CTL in controlling viral replication and delaying disease progression (Table I).

1.5.1 *Temporal changes in anti-HIV CTL activity*

Studies of CTL activity at different stages of disease provide some evidence of a role for HIV-specific CTL in controlling HIV replication. During primary infection, plasma HIV RNA rapidly rises to extremely high levels, CD4⁺ T cell numbers fall and CD8⁺ T cell numbers increase [40,41]. HIV-specific CTL become detectable and plasma virus load falls by several orders of magnitude before neutralizing antibodies appear [42]. In two studies of CTL responses in primary infection, one of five individuals in each study made no detectable CTL response [43,44]. Both these individuals had consistently high virus loads and rapidly progressed to AIDS. Therefore, in addition to resolving the viremia of acute infection, early generation of an effective HIV-specific CTL response may dramatically influence subsequent disease course.

One study found that generation of CTL against HIV proteins (rev and tat) involved in early viral replication was of particular importance in protection against rapid disease progression [45].

Table I. Evidence supporting a protective role for HIV-specific CTL

<i>Evidence</i>	<i>References</i>
CD8 ⁺ T cell depletions in SIV-infected macaques	Schmitz, 1999 [38]; Jin, 1999 [39]
Temporal changes in anti-HIV CTL	Borrow, 1994 [43]; Moss, 1995 [49]; Gamberg, 1999 [51]
HIV-specific CTL in long-term non-progressors	Betts, 1999 [54]; Wagner, 1999 [55]; Harrer, 1996 [56]
HIV-specific CTL in exposed uninfected individuals	Fowke 1996, [60]; Bernard, 1999 [61]; Rowland-Jones, 1995 [68]
Correlation of HIV-specific CTL and markers of disease progression	Greenough, 1997 [70]; Lubaki, 1999 [72]; Ogg, 1999 [76]
Association between HLA type and disease progression	Hill, 1996 [78]; Hendel, 1999 [80]; Carrington, 1999 [83]
Occurrence of CTL escape mutants	Goulder, 1997 [13]; Borrow, 1997 [89]; Price, 1997 [90]; Evans, 1999 [92]

Studies of the TCR repertoire in primary HIV infection show that the CD8⁺ T cell response is often comprised of large (up to 40% of all T cells in the peripheral blood), but transient oligoclonal T cell expansions [46]. Although this type of response has also been described in acute infectious mononucleosis and may occur in other acute viral infections, it signifies a particularly poor prognosis in HIV infection [46,47]. Thus, in addition to or even more so than its strength, the breadth of the early anti-HIV CTL response may be an important determinant of longer-term clinical outcome.

Throughout the asymptomatic period of HIV infection, the anti-HIV CTL response appears stable, with HIV-specific CTL frequency estimated at up to 1% of circulating T cells [48,49]. This vigorous CTL response results from continuous antigenic stimulation by a virus constantly replicating in multiple sites [50]. As disease progresses, plasma virus load increases significantly and HIV-specific CTL activity appears to weaken [51]. In advanced HIV infection, CD8⁺ T cell numbers fall dramatically and effector CTL virtually disappear [51]. This consistent pattern of decaying HIV-specific CTL responses over the course of disease progression provides circumstantial evidence that anti-HIV CTL play a role in suppressing viral replication and delaying disease progression.

1.5.2 *HIV-specific CTL in long-term nonprogressors*

An extraordinarily benign disease course occurs in a small proportion of HIV-infected individuals termed long-term nonprogressors (LTNP). This heterogeneous group is thought to consist mostly of very slow progressors with very few true nonprogressors. Several studies have shown strong persistent HIV-specific CTL responses in LTNP and it

has been proposed that HIV-specific CTL from LTNP may have distinct features responsible for prolonging viral suppression and the asymptomatic phase of infection [52-54]. The CTL responses of LTNP generally involve simultaneous recognition of multiple epitopes and, in some cases, target conserved HIV sequences essential for viral replication [12,55,56]. CTL precursor frequencies are also reportedly higher for LTNP than for intermediate or rapid progressors [53,57]. Since neutralizing antibody activity is often weak or absent in these individuals, this implicates qualitative and quantitative aspects of the CTL response more so than humoral immunity in the immune system's contribution to long term nonprogression of HIV infection [53,57].

1.5.3 *Anti-HIV CTL in exposed uninfected individuals*

A small fraction of individuals exposed to HIV through sexual contact, intrauterine exposure, or occupational exposure resist infection. Such resistance could result from genetic and/or acquired factors. Several years ago, a certain degree of natural resistance to HIV was demonstrated to occur through inheritance of a defective form of the HIV co-receptor CCR-5 [58,59]. Homozygosity for a 32 base pair deletion mutation at the CCR-5 locus prevents expression of the gene product, virtually abolishing susceptibility to infection by viruses dependent upon CCR-5 as a co-receptor. However, the homozygous CCR-5 Δ 32 genotype accounts for only a small percentage of the individuals who resist HIV infection despite repeated exposure [60,61]. Other genetic factors have been identified that delay disease progression but have little or no effect on HIV transmission. These include heterozygosity for the CCR-5 Δ 32 mutation, a CCR-2

co-receptor mutation (CCR-2 64I), and an SDF1 chemokine gene variant (SDF1-3A) [62-64]. Although these factors may contribute to long-term nonprogression in certain cases, they do not appear to be responsible for the instances of apparent resistance to infection that have been described.

There is now considerable evidence supporting the idea that resistance to infection is acquired through induction of HIV-specific immunity. HIV-specific CD8⁺ CTL activity in the absence of seroconversion has been demonstrated for some individuals in each of the exposed uninfected groups discussed above [65-68]. Furthermore, low dose immunization of macaques with a live SIV vaccine induces specific cell-mediated immune responses without humoral immunity. Macaques immunized in this way are protected from challenge with normally infectious doses of virus [69]. These data demonstrate that, in some cases, initial exposure to small amounts of an AIDS-causing retrovirus induces cell-mediated immunity associated with protection from infection upon subsequent exposure to infectious doses of the virus.

1.5.4 Relationships between anti-HIV CTL activity and surrogate markers of disease progression

Many studies have addressed the association between HIV-specific CTL activity and disease progression. A number have reported negative correlations between HIV-specific CTL activity and virus load [54,70-73] and positive correlations between HIV-specific CTL activity and CD4⁺ T cell counts or proliferative responses [70,71,73,74]. In two studies using TCR-specific tetramers to quantitate HLA-A2-restricted CTL against gag, there was a significant association between the frequency of tetramer-binding cells

and disease stage [75,76]. These data suggest that stronger HIV-specific CTL responses confer better control of viral replication, resulting in a slower loss of CD4⁺ T cells and delayed disease progression.

1.5.5 *Associations between HLA type and rates of disease progression*

If CTL are important in controlling HIV infection, HLA class I type should be a key factor in determining the rate of disease progression. Selection of epitopes is dependent on HLA type and selection of more conserved HIV sequences as CTL epitopes could contribute to longer-term control of viral replication. Although more than 50 reports examining the contribution of HLA type towards the outcome of HIV infection have been published, the associations reported have generally been weak and not always corroborated. This is largely due to the extreme polymorphism of the HLA complex as there are currently more than 300 known alleles at the HLA class I A, B and C loci [77]. Thus, many individual alleles are relatively rare, making associations difficult to observe [78,79].

A recent study of the association between HLA type and disease progression within the “genetics of resistance to infection by HIV” (GRIV) cohort tries to overcome this limitation [80]. GRIV is a cohort of well characterized individuals representing the extremes of rapid progression and nonprogression. This cohort currently consists of 200 slow progressors and 75 rapid progressors, but because the definition of slow progression captures ~1% of HIV infected individuals, this study essentially draws on a cohort of 20,000 individuals. Previously reported associations of HLA-B27 and B57 with slow progression and of HLA-B35 with rapid progression to AIDS were confirmed in this

study [81,82]. In addition, the “GRIV” study reported that several other HLA alleles, not previously associated with disease progression affect progression to AIDS more strongly. HLA-B14, Cw14, and Cw8 were found to be highly protective, while HLA-A29, B22, and Cw16 conveyed an increased risk of rapid progression. This study confirmed an important role for HLA in affecting the rate of HIV disease progression, with the protective effect of certain HLA alleles comparable with that afforded by heterozygosity for the CCR-5 Δ 32 mutation.

Another recent study took a different approach to determining whether HLA type affects the rate of disease progression. In this report, survival and genetic association analyses were performed on individuals enrolled in five AIDS cohorts to determine whether overall or specific locus heterozygosity at the HLA class I loci confers relative resistance to AIDS progression [83]. Maximum heterozygosity at the HLA class I-A, B, and C loci was associated with slower progression to AIDS and death compared to individuals homozygous for one or more loci. Presumably, this is because heterozygosity allows presentation of a broader range of HIV peptides and potentially generates a more diverse immune response.

1.5.6 *Viral mutants that escape CTL recognition*

The rapid rate of HIV replication combined with the high error rate of reverse transcriptase produces an extremely high mutation rate for HIV [84,85]. Within an HIV-infected individual, this produces vast numbers of mutants. However, only those specific mutations conferring an advantage to the virus selectively accumulate [86]. Therefore, if

CTL are important for controlling HIV infection, it is reasonable to speculate that mutations in CTL epitopes that serve as a mechanism of immune escape will be selected. Considerable evidence has been gathered suggesting that escape from CTL recognition plays a role in HIV persistence [87,88]. CTL escape mutants emerge in primary infection and, in two cases, immune escape after 9-12 years of asymptomatic infection was associated with rapid progression to AIDS [13,89,90]. When an autologous ex-vivo expanded nef-specific CTL clone was adoptively transferred back into the donor, viral escape mutants lacking the specific sequence recognized by the CTL quickly emerged and the patient rapidly progressed to AIDS [91]. In this case, there is also a possibility that the mutation in nef increased the virulence of the virus, but it nonetheless indicates that HIV-specific CTL can control viral replication in vivo and exert selective pressure on the viral quasispecies present.

Perhaps the most definitive evidence for an association between HIV mutational escape of CTL recognition and disease progression comes from a recent study in the SIV system [92]. The specificity of anti-SIV CTL was determined in SIV-infected macaques early in infection and selective pressure for mutations within CTL epitope sequences was compared with other viral sequences. The authors observed selective accumulation of mutations within viral CTL epitopes in concert with loss of immunological control of viral replication. Mutational escape from CTL recognition is discussed in greater detail below.

1.6 Why anti-HIV CTL ultimately fail

If anti-HIV CTL do delay disease progression, they clearly do not mediate lifelong protection from progression to AIDS. A key unanswered question is how HIV manages to persist, replicate and eventually cause disease despite a vigorous anti-HIV CTL response. A number of mechanisms have been proposed to explain this apparent paradox, but no consensus as to the *in vivo* relevance of any of these mechanisms has emerged (Table II).

1.6.1 *Exhaustion of HIV-specific CTL*

In some cases of advanced HIV infection, neither circulating nor inducible HIV-specific CTL activity is detectable, suggesting that the repertoire of effective anti-HIV CTL may have been depleted by clonal exhaustion [51]. Clonal exhaustion of an antiviral CTL response was previously demonstrated in mice infected with high doses of a rapidly replicating strain of LCMV [93]. A similar situation may arise in HIV infection, where prolonged activation and expansion of HIV-specific CD8⁺ T cells could push these cells to the limit of their replicative lifespan [94,95]. The CD8⁺ T cells of HIV infected individuals have significantly shorter telomeres than those of age-matched non-infected controls indicating a history of extensive cell division, limited replicative reserve and possible proximity to clonal exhaustion [96,97]. This is particularly true for the CD8⁺CD28⁻ T cell subset, which markedly expands following HIV infection. In primary infection, exhausted HIV-specific CTL clones are supplanted by new dominant clones, but if exhaustion were to occur at a later stage of disease, with T cell number and

Table II. Possible mechanisms for the ultimate failure of HIV-specific CTL

<i>Mechanism</i>	<i>References</i>
Exhaustion of HIV-specific CTL	Wolthers,1998 [96]; Effros,1996 [97]; Pantaleo,1997 [98]
Apoptosis of HIV-specific CTL	Xu,1997 [104]; Tan,1999 [105]; McKinney,1999 [106]
Loss of CD4 ⁺ T cell help	Fauci,1996 [109]; Rosenberg,1997 [110]; Pitcher, 1999 [111]
Antigenic variation	Haas,1996 [118]; Price,1998 [119]; Evans, 1999 [92]
Down regulation of surface HLA expression	Kerkau,1989 [125]; Schwartz,1996 [129]; Collins,1998 [130]

function more severely compromised, it might be impossible for new responses to arise [98].

1.6.2 *Apoptosis of HIV-specific CTL*

Abnormally high levels of apoptosis occur in the CD4⁺ and CD8⁺ T cell populations of HIV-infected individuals [99]. Much of this apoptosis may be related to overexpression of fas and fas ligand. Infection of CD4⁺ T cells or macrophages with HIV upregulates fas ligand expression and the proportion of CD4⁺ and CD8⁺ T cells expressing fas also increases in HIV infection [100-102]. This raises the possibility that HIV-infected cells could kill HIV-specific CTL they encounter, in a manner similar to that proposed for the killing of tumor-specific T cells by fas ligand-expressing malignant cells [103]. Macaques infected with an attenuated SIV strain encoding a defective nef protein developed strong SIV-specific CTL responses, whereas animals infected with a pathogenic SIV strain had low to absent SIV-specific CTL responses [104]. The investigators in this study then demonstrated that while infection of CD4⁺ T cells in vitro with the pathogenic strain of SIV upregulated fas ligand expression, the nef-defective strain had no such effect. Upregulation of fas ligand expression by nef in infected cells could favor apoptosis of CD8⁺ anti-HIV CTL and provide an explanation for the role of HIV and SIV nef in the pathogenesis of AIDS. Further support for this possibility was provided by Tan et al., who showed that adoptively transferred autologous in vitro expanded HIV-specific CTL clones were eliminated by apoptosis within hours of infusion [105]. McKinney et al. transferred HIV-specific CTL clones into HIV infected and uninfected human-PBL-SCID mice and showed that the HIV-specific CTL rapidly

disappeared in infected recipients, while they persisted at high levels in the uninfected mice [106]. These studies support the possibility that increased apoptosis of HIV-specific CTL contributes to the decline in CTL activity observed in late stage HIV infection.

1.6.3 *Loss of CD4⁺ T cell help*

Although disappearance of CTL may account for some cases of disease progression, vigorous and sometimes diverse anti-HIV memory CTL activity can be sustained even in individuals with full-blown AIDS [51,107,108]. In these cases, circulating CTL responses are usually undetectable, while anti-HIV CTL activity remains inducible *in vitro*. Therefore, an *in vivo* defect in the activation of anti-HIV CTL memory cells appears to render these cells ineffective in advanced HIV infection.

In our view, it is most probable that loss of CTL activity at this stage is secondary to the absolute loss of CD4⁺ T cells and impaired function of those remaining. It has been reported that even relatively early in HIV infection, HIV-specific CD4⁺ T cell proliferation becomes undetectable in most infected individuals [109,110]. Recently, however, flow cytometry-based methods to detect intracellular cytokine (γ -interferon) production have shown that HIV-specific CD4⁺ T cells persist in all individuals with nonprogressive infection and in most subjects with active, progressive infection [111]. Therefore, although proliferative responses to HIV appear to be lost at an early stage of infection, functional HIV-specific CD4⁺ cells that secrete γ -interferon following stimulation with HIV antigens do persist into the chronic phase of infection. It is still

unclear how this disjunction of proliferation and cytokine production relates to HIV-specific helper T cell function in vivo. Although the CD8⁺ T cell-mediated rejection of genetically modified HIV-specific CTL clones by HIV infected individuals with low CD4 counts indicates the preserved ability to generate CTL responses in advanced HIV infection, long-term maintenance of these CTL responses is another question [112]. Depletion of CD4⁺ T cells by antibody treatment did not impair the primary CTL response to LCMV in mice, but the mice failed to maintain CTL memory during persistent infection [113]. If maintenance of CTL memory depends on CD4⁺ T cell help, declining CTL activity in progressive HIV infection appears inevitable in the wake of severe CD4⁺ T cell depletion.

1.6.4 *Effects of antigenic variation*

The several well-documented examples of HIV mutational escape from CTL discussed above share two important features [13,89-91]. In each case, there was a dominant CTL response to a single epitope, and the mutation, which was selected to fixation, disrupted binding of the peptide to the presenting HLA molecule. Restriction of the CTL response to a single epitope in these individuals made escape relatively easy, as a single mutation allowed complete evasion of the CTL response. However, as previously described, most HIV-infected individuals mount polyclonal CTL responses involving recognition of multiple HIV epitopes [10,11]. In a related model, the murine CTL response to a single hepatitis B virus (HBV) epitope was analyzed [114]. Diverse polyclonal responses were generated and no single mutation in this epitope ever affected all the responding T cell clones. Therefore, in most cases, mutational inactivation of a

single epitope is highly unlikely to be sufficient for viral evasion of the immune system. Simultaneous complementary mutations would be required and, until recently, simultaneous complementary mutations at more than two epitope sites was considered rare [85]. A recent study by Evans demonstrated that, in SIV-infected macaques with up to five concomitant CTL responses, viral mutation and immune selection produced dominant viral quasispecies harboring mutations within all five epitope sites [92]. This selection was associated with increased levels of viral replication and disease progression. Therefore, mutational escape from CTL recognition during HIV infection may be more widespread than previously anticipated.

Antigenic variation can affect the efficiency of the CTL response even without producing complete mutational escape from CTL recognition. At any one time within an infected individual, swarms of related but genetically distinct viruses exist as proviruses and virions. In many patients with concurrent CTL responses to multiple epitopes, mutations that would affect the CTL response exist within proviral quasispecies [115,116]. In an HIV-infected individual with CTL responses to two epitopes in *gag*, the CTL response was unstable over time as it switched back and forth between epitopes [117]. There were concurrent fluctuations in the predominant virus sequence present, with escape variants for each epitope increasing in frequency when the corresponding CTL response was strong and decreasing as it weakened. Another study of four asymptomatic individuals showed that particular variants dominated the viral quasispecies for only short periods of time and were eliminated by HIV-specific CTL [118]. Thus, the CTL response and viral variants existing at any one time in an HIV-infected person may reflect an extended series of past escapes and selections.

Mutations within CTL epitopes that don't affect binding to HLA molecules nonetheless have the potential to subvert CTL recognition. In such cases, a so-called altered peptide ligand (APL) is presented to the T cell, which can profoundly affect T cell recognition and subsequent activation of CTL effector functions [reviewed in 119]. APL may act simply as escape mutants which, although potentially immunogenic, are not recognized by the CTL response existing in the peripheral blood of an individual at a given time [120,121]. Alternatively, APL may actually antagonize the existing CTL response. In this case the APL has a partially productive interaction with the TCR, which through a poorly understood intracellular signaling mechanism, prevents the CTL from lysing even those cells infected with wild-type virus [116,122,123]. Partially recognized APL can also favor the virus by continuing to stimulate the expansion of CTL no longer effective at controlling viral replication. In the LCMV model, partial reactivity of variant peptides with pre-existing CTL inhibits the recruitment of CTL that can more efficiently recognize the variant [124]. These dual potential actions of APL to perpetuate an ineffective CTL response and functionally silence reactive CTL may give the virus a very significant survival advantage.

1.6.5 *Down-regulation of HLA class I molecules*

Downregulation of HLA expression on infected cells is a common potential means of viral escape from CTL. Several earlier studies showed that HIV could decrease surface expression of HLA molecules on infected cells without examining the impact of this downregulation on recognition by HIV-specific CTL [125,126]. Previously, HIV tat and/or vpu were thought to mediate this effect, but more recently, nef was found to

induce endocytosis of HLA class I molecules [127-129]. A separate study showed that target cells infected with a nef-deleted HIV mutant did not alter HLA expression and were fully susceptible to CTL mediated lysis, whereas cells infected with wild-type HIV were relatively resistant to lysis and showed up to 20-fold downregulation of HLA class I expression [130].

The significance of these findings for the ability of HIV-specific CTL to control viral replication in vivo is unclear. This avoidance mechanism must be incomplete because very strong CTL responses are induced and persist in nearly all infected individuals. However, with the high levels of HIV replication that occur, any reduction in the efficiency with which CTL recognize infected cells could have a substantial overall effect on virus production and the rate of disease progression.

1.7 Anti-HIV CTL in the era of highly active antiretroviral therapy (HAART)

Triple combination antiretroviral drug regimens have dramatically reduced the incidence of HIV-related morbidity and mortality [131]. Even in advanced HIV infection, HAART can effectively suppress HIV replication and increase circulating T cell numbers [132,133]. However, clinical reports of severe opportunistic infections, despite satisfactory responses to antiretroviral treatment, suggest that increased T cell counts may not always signify functional augmentation of the immune system [134]. Previous studies demonstrated HAART-related improvements in CD4⁺ T cell function as indicated by increased lymphoproliferative responses to mitogens and recall antigens, but HIV-specific CD4⁺ T cell responses did not improve in the same individuals [133,135]. Therefore, despite overall improvements in CD4⁺ T cell function with HAART, it doesn't

seem that HIV-specific CD4⁺ responses are reconstituted. However, if individuals are treated early enough with HAART, there is some evidence that CD4⁺ T cell responses to HIV are preserved and that this favors stronger HIV-specific CTL responses over the longer term [110].

Studies using peptide/MHC tetrameric complexes to enumerate the frequency of HIV peptide-specific CD8⁺ T cells in individuals treated with HAART have shown that anti-HIV CTL decline in number with effective suppression of viral replication [136-138]. In all cases, the frequency of tetramer-binding cells declined in patients responding to therapy. Most studies also show that the functional activation of anti-HIV CTL declines with potent suppression of viral turnover [136,137,139]. In a detailed longitudinal evaluation of two individuals before and after the start of combination therapy, both subjects had in vivo activated and memory CTL against multiple HIV gene products before starting therapy [136]. Effective therapy reduced the levels of in vivo activated HIV-specific CTL, but concurrently increased the frequency of HIV-specific memory CTL. Prolonged suppression of viral replication also reduced levels of memory CTL. Over the course of study, both subjects had transient increases in viral load associated with increased levels of virus-specific CTL, suggesting the immune system can still respond rapidly when memory CTL fall to low levels. In two other reports, anti-HIV CTL activity was unchanged by antiretroviral therapy [140,141]. In our laboratory, we saw reconstitution of circulating CTL activity and inducible HIV-specific CTL activity in two out of three subjects with advanced HIV infection who responded to HAART with viral suppression and increased T cell counts [unpublished data]. This demonstrates that if antiretroviral therapy is effective, even the residual CD8⁺ T cell

population persisting through advanced HIV infection can reconstitute functional HIV-specific CTL responses.

Studies examining changes in the phenotype of CD8⁺ T lymphocytes in response to HAART report reduced numbers of activated CD8⁺ T cells (CD38⁺DR⁺CD28⁻), consistent with declining CTL activity [136,140,142]. However, the percentage of activated CD8⁺ T cells in these individuals remained significantly elevated compared with uninfected controls, suggesting either ongoing occult HIV replication or some other form of chronic stimulation of CD8⁺ T cells [142].

In conclusion, HAART reduces the number of activated CD8⁺ T cells and HIV-specific CTL activity. Since the nature of the immune system is to respond to invading pathogens, it is not surprising that as the antigenic burden is reduced by HAART, HIV-specific CTL responses wane. The observations of rapid CTL responses when virus load increases, as well as reconstitution of HIV-specific CTL responses when T cell numbers increase, indicate that the CD8⁺ T cell population largely remains intact throughout progressive HIV infection. The recent demonstration of continuous thymic T cell production throughout adult life and of increased thymic output in HIV-infected individuals effectively treated with HAART also supports the possibility of reconstituting effective CTL responses [143]. However, because the frequency of HIV-specific CTL rapidly declines with effective treatment, therapeutic vaccination as an alternative to treatment interruptions should be considered to strengthen the HIV-specific CTL responses of individuals being successfully treated with HAART. If such an approach reduces dependence on antiretroviral drugs for suppression of HIV replication below

detectable levels, this will clearly establish a contribution from anti-HIV CTL to protection from disease progression.

1.8 Summary

The basic question of whether HIV-specific CTL are protective or not remains unanswered. There is circumstantial evidence that some exposed uninfected individuals generate HIV-specific CTL responses that protect them from infection. Evidence of selective pressure exerted on HIV quasispecies by anti-HIV CTL demonstrates that the CTL response can impact on viral replication in vivo. Associations between particular HLA class I alleles and rates of disease progression strongly suggest that HLA class I-restricted T cell responses influence viral replication. The decay of anti-HIV CTL activity in parallel with disease progression is also consistent with a role in delaying disease progression. HIV-specific CTL responses appear variable in their ability to control HIV replication and qualitative differences in CTL responses are probably responsible for this. Restricted CTL responses against HIV appear particularly ineffective for the long-term control of viral replication, but in some individuals, even strong diverse anti-HIV CTL responses do little to limit viral replication. Extensive studies of the timing, epitope specificity and phenotype of anti-HIV CTL are required, together with relevant clinical follow-up to establish what CTL characteristics favor effective long-term suppression of viral replication.

This review also discussed potential mechanisms for the failure of CTL responses in progressive HIV infection. Multiple mechanisms are speculatively implicated in contributing to the loss of CTL activity in advanced infection, but there is no consensus

as to which mechanism, if any, predominates. Both accelerated apoptosis and clonal exhaustion may drive absolute depletion of HIV-specific CD8⁺ T cells, while generation of escape mutants and APL reduces the functional efficiency of those remaining. Productive recognition of target cells by CD8⁺ anti-HIV CTL may also be impaired by reduced expression of HLA class I antigens and up-regulation of fas ligand on HIV-infected cells. Chronic activation of CD8⁺ T cells subsequent to their failure to eliminate HIV probably produces secondary functional deficits reflected in loss of CD28 expression, down-regulation of TCR signaling components and expression of KIR. The possibility of reshaping anti-HIV CTL responses with therapeutic vaccines while HIV is suppressed by HAART may allow definition and demonstration of protective CTL responses against HIV.

1.9 Thesis Proposal and Specific Research Objectives

When this thesis project was first proposed, the role of HIV-specific CTL in the pathogenesis of HIV infection was highly equivocal. Although the majority of studies supported the concept that anti-HIV CTL contributed to controlling viral replication and delaying the onset of disease, several other observations suggested that HIV-specific CTL were deleterious to the infected host, mediating CD4⁺ T cell depletion and progression to AIDS. Since then, a central antiviral role for CD8⁺ T cells in SIV infection has been clearly demonstrated, and it is now widely accepted that HIV-specific CTL play a similar protective role in HIV infection. However, it is also clear that anti-HIV CTL *per se* do not mediate lifelong protection from progression to AIDS. Current data strongly points to multiple mechanisms contributing to the failure of anti-HIV CTL to control HIV infection indefinitely. HIV-specific CTL appear variable in their ability to slow the rate of disease progression, but the exact determinants involved have not yet been revealed. The primary purpose of this thesis was to further elucidate the biological relevance of anti-HIV CTL, by characterizing the CD8⁺ T cell response during different clinical stages of HIV disease. Understanding CD8⁺ T cell regulation is critical for understanding immune pathogenesis and for developing strategies for immune intervention for HIV and other diseases.

The specific research objectives of this thesis were:

- (i) to investigate the functional and genetic characteristics of the CD8⁺ T cell population in individuals with advanced HIV infection.

- (ii) to determine the potential for reconstitution of CD8⁺ T cell immunity in individuals with advanced HIV infection.
- (iii) to examine whether the expanded CD8⁺CD28⁻ T cell population from individuals at various stages of HIV infection contributes to disease progression
- (iv) to identify virological and immunological parameters affecting the maintenance of HIV-specific memory T cell responses over prolonged periods of HIV infection.

To achieve these objectives two major studies were performed. The first involved a detailed examination of the CD8⁺ T cell population persisting through the advanced stages of HIV infection. It was hypothesized that, by defining CTL characteristics peculiar to advanced infection, where cumulative defects have been imposed on the CD8⁺ T cell population, reasons underlying the ultimate failure of anti-HIV CTL responses might be revealed. During this study several individuals responded to antiretroviral therapy, evidenced by control of viral replication to undetectable levels and partial reconstitution of T cell lymphopenia. These subjects were examined for an extended period, to determine whether intractable defects precluded HIV-specific immune responses at this stage, or if, even following advanced infection, immune reconstitution enabled specific immune responses against HIV.

The second study involved a larger group of HIV-infected individuals, who were at various stages of infection. This group was studied to determine whether the expanded CD8⁺CD28⁻ T cell population arising during HIV infection reflects the CD8⁺ T cell

dysfunction that occurs with disease progression, and to identify phenotypic characteristics that could predict persistence or degeneration of anti-HIV CTL.

This thesis is presented in manuscript format, with each chapter representing a 'stand alone' research paper. The following four chapters consecutively describe the experimental procedures and results related to the specific objectives outlined above. The final chapter represents an integrated discussion on the thesis as a whole.

1.10 References

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CHAPTER 2

Functional and Genetic Integrity of the CD8⁺ T Cell Repertoire in Advanced HIV Infection¹

Running head: CD8⁺ T Cells in Advanced HIV Infection

text = 4422 words

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¹This work was supported by grants from the Canadian Foundation for AIDS Research (CANFAR), the National Health Research Development Program (NHRDP) and the Medical Research Council (MRC) of Canada. MDG is a recipient of an MRC AIDS scholar award. JCG is a recipient of a Memorial University of Newfoundland graduate fellowship.

2.1 Abstract

Background: HIV-specific cytotoxic T lymphocytes (CTL) can restrict HIV replication in acute and chronic infection, but disease progression occurs in parallel with declining CTL activity. Understanding why CTL fail to control HIV replication might reveal important mechanisms of disease progression and enhance prospects for developing effective CTL-based immunotherapies.

Objectives: To investigate the functional integrity, T cell repertoire diversity, and HIV reactivity of CD8⁺ T lymphocytes in individuals with advanced HIV infection.

Methods: Individuals were considered to have progressed to advanced HIV infection if their total T cell count was <500/ μ l blood on at least two successive clinic visits. CD8⁺ T cells from these individuals were analyzed for CTL function, HIV reactivity and T cell receptor (TCR) diversity by chromium release assays and reverse transcriptase polymerase chain reaction (RT-PCR).

Results: CD8⁺ T cells from all individuals with advanced HIV infection proliferated and differentiated into functional CTL *in vitro*. Despite extremely low T cell counts and previous AIDS defining illnesses, 6 individuals had inducible anti-HIV CTL responses. In two additional cases, HIV-specific CTL activity became detectable following significant treatment-associated remission of T cell lymphopenia. Assessment of TCR β V gene family representation and β V gene intrafamily diversity indicated CD8⁺ T cell repertoire diversity is maintained through advanced HIV infection.

Conclusions: These data suggest that HIV-specific CTL activity can be selectively compromised while the functional and genetic integrity of the CD8⁺ population as a whole remains intact. A substantial fraction of individuals retain inducible anti-HIV CTL

activity through advanced HIV infection and, in at least some cases, effective treatment can restore HIV-specific CTL responses even at this late stage of disease.

Keywords: Human immunodeficiency virus (HIV), cytotoxic T lymphocytes (CTL), T cell receptor (TCR) repertoire.

2.2 Introduction

There is considerable evidence that CTL-mediated control of HIV replication delays disease progression. HIV-specific CTL arise during primary HIV infection and typically persist throughout the clinically asymptomatic period [1, 2]. The appearance of HIV-specific CTL coincides with decreasing viremia, and anti-HIV CTL inhibit viral replication and eliminate virus-infected cells *in vitro* [3-8]. Strong HIV-specific CTL responses occur in long-term non-progressors and in a significant fraction of HIV-exposed uninfected individuals [9-12]. Anti-HIV CTL diversity in primary infection predicts slow disease progression and emergence of CTL-escape mutants sometimes precedes rapid progression to AIDS [1, 13-15].

Untreated HIV infection ordinarily progresses to AIDS, despite vigorous CTL responses. Over the transition to advanced infection, HIV-specific CTL activity weakens as total CD8⁺ T cell numbers fall [16-18]. Persistence of anti-Epstein-Barr virus (EBV) CTL and general CTL activity, while anti-HIV CTL disappear, suggests selective loss of HIV-specific CTL [16, 19, 20]. Signs of cellular senescence such as proliferation defects and short telomeres occur in CD8⁺ T cells from HIV-infected individuals, particularly within the disproportionately expanded CD28⁻ subset [21, 22]. At least two non-exclusive theories address CTL decline in HIV infection: (1) depletion through clonal exhaustion or apoptosis [23-27]; and (2) anergy imposed through inadequate T cell help or inappropriate antigen presentation [28-31]. Defining CTL characteristics peculiar to advanced infection may reveal the relevance of these and other mechanisms to CTL failure. This information is important for understanding disease progression and

evaluating prospects for CTL-based immunotherapy and reconstitution of CD8⁺ T cell immunity.

It is presently uncertain whether HIV-specific precursors, or even normal functional attributes of CD8⁺ T cells, persist through advanced infection [32]. While primary infection is associated with oligoclonal expansions within TCR β chain variable gene (TCR β V) families, the status of TCR repertoire diversity throughout chronic infection is unclear [13, 33, 34]. More research is needed to determine whether CD8⁺ T cells in advanced HIV infection comprise a functionally and genetically intact population with residual immune potential against HIV and other pathogens.

We examined CD8⁺ T cells from 13 individuals with advanced HIV infection, for proliferative potential, CTL function, HIV reactivity and TCR diversity. Although we rarely detected circulating CTL, CD8⁺ T cells from all 13 individuals proliferated and differentiated into functional CTL *in vitro*. Anti-HIV CTL were initially detected in 6/12 individuals, but they became detectable following treatment-associated remission of T cell lymphopenia in 2 additional cases. While CD8⁺ T cells in the circulation and following *in vitro* stimulation were predominantly CD28⁺, the anti-HIV CTL we detected in this group were predominantly CD8⁺CD28⁻. Relative TCR β V gene family expression levels and intrafamily diversity largely mirrored the range exhibited by uninfected controls. These results suggest that over the course of HIV infection, HIV-specific CTL activity deteriorates while the functional and genetic integrity of the CD8⁺ population generally remains intact.

2.3 Methods

2.3.1 Study subjects

HIV-infected individuals were recruited through the Infectious Disease Clinic of the St. John's General Hospital, St. John's, NF, Canada. Individuals presenting with total T cell counts $<500/\mu\text{l}$ peripheral blood on at least two consecutive clinic visits were considered to have progressed to advanced HIV infection and recruited for this study. At each visit, clinical evaluation was performed and blood was drawn for measurement of plasma HIV RNA using Amplicor HIV-1 MonitorTM quantitation kits (Roche Diagnostic Systems Inc., Mississauga, ON), for measurement of peripheral blood lymphocyte (PBL) subsets and for our research study. Ethical approval was obtained from the Memorial University Faculty of Medicine Human Investigation Committee and informed consent for drawing blood samples and accessing medical records was obtained from all participants. Uninfected controls were laboratory or hospital personnel.

2.3.2 Sample preparation

Heparinized whole blood samples were processed within 4 hours of collection. Samples were centrifuged at 500g for 10 minutes and plasma was removed and immediately frozen at -70°C . Packed blood was diluted to twice the original volume with sterile phosphate buffered saline (PBS), transferred to 50 ml centrifuge tubes and underlaid with an approximately equal volume of Ficoll-paque lymphocyte separation medium (Pharmacia Chemicals, Dorval, Quebec). After centrifuging for 30 minutes at 400g, the

peripheral blood mononuclear cell (PBMC) layer was collected, washed twice in PBS with 1% fetal bovine serum (FBS; Gibco, Grand Island, NY), resuspended in lymphocyte medium (RPMI 1640 with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2×10^{-5} M 2-mercaptoethanol; all from Gibco) and counted. Freshly-isolated cells were used for HLA typing, B lymphoblastoid cell line (BLCL) generation, determining circulating CTL activity and determining the proportion of $CD8^+$ T cells expressing CD28. A portion of the freshly-isolated cells were stimulated *in vitro* prior to determining anti-HIV CTL activity and TCR repertoire status. Class I human histocompatibility-linked antigen (HLA)-A and B antigens were determined using standard serologic techniques [35]. BLCL were generated by infecting 5×10^6 PBMC with 2.5 ml supernatant from the B95-8 cell line obtained from the American Type Culture Collection, (ATCC CRL1612, Rockville, MD). After 24 hours, cells were pelleted and resuspended in lymphocyte medium containing 20% FCS and 1 μ g/ml cyclosporin A. Transformation usually occurred after 2-3 weeks, and cell lines were preserved in liquid nitrogen.

2.3.3 Lymphocyte stimulation

Approximately 1×10^7 freshly-isolated PBMC were stimulated by an autologous mixed lymphocyte reaction (AMLR), similar to that previously described [36]. Briefly, 90% of the cells (responders) were cultured in plain lymphocyte medium, with the remaining 10% (stimulators) cultured in lymphocyte medium supplemented with 5 μ g/ml purified phytohemagglutinin (PHA-P; Wellmark Diagnostics, Guelph, ON) and 10 U/ml recombinant human interleukin-2 (IL-2; Hoffmann La Roche, Nutley, NJ). After 3 days,

the stimulators were washed twice with lymphocyte medium and added to the responder population. Three days later, 5 U/ml IL-2 was added, and culture continued for an additional 7 days, with addition of lymphocyte medium supplemented with 5 U/ml IL-2 as required.

2.3.4 Flow cytometry

To determine the proportion of CD8⁺ T cells expressing CD28, cells were washed once in PBS containing 5 mM EDTA and 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and incubated for 20 minutes at 4 °C with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (Dako Diagnostics, Mississauga, ON), phycoerythrin (PE)-conjugated anti-CD28 (Becton Dickinson, Mississauga, ON) and Peridinin Chlorophyll Protein (PerCP)-conjugated anti-CD8 (Becton Dickinson). Samples were washed once after staining, resuspended in 0.5% paraformaldehyde in PBS and analyzed on a FACStar^{Plus} analyzer (Becton Dickinson) after excitation at 488 nm with an argon laser.

2.3.5 Lymphocyte separations

To remove CD28⁺ cells before cytotoxicity assays, cells were pelleted, washed in PBS with 5 mM EDTA and 0.1% BSA and incubated for 20 minutes at 4 °C with 5 µg/10⁶ cells of purified anti-CD28 (Becton Dickinson). Cells were washed once more and then incubated at 3x10⁶/ml in PBS with 5 mM EDTA and 0.1% BSA at 4 °C for 45 minutes on a rotating mixer with goat-anti-mouse IgG coated magnetic beads (Dynal Inc., Great Neck, NY) at a 10:1 bead-to-target cell ratio. Bound cells were separated by holding the tube against a magnet, removing the supernatant, washing the beads gently in PBS and

repeating the process. For depletion studies the unbound cells were centrifuged and resuspended based on starting cell numbers for use as effectors in cytotoxicity assays. For enrichment studies, the unbound cells were counted and effector:target (E:T) ratios re-established with purified cells. Flow cytometry revealed that this depletion method removed >98% of CD28⁺ cells (data not shown).

For TCR repertoire analysis CD8⁺ T cells were isolated from the stimulated cultures. Cells were pelleted, washed, and incubated at 3×10^6 /ml in PBS with 5 mM EDTA and 0.1% BSA at 4 °C for 45 minutes on a rotating mixer with anti-CD8 coated magnetic beads (Dynal Inc.) at a 10:1 bead-to-target cell ratio. Bound cells were separated as described above and frozen at -70 °C for RNA extraction.

2.3.6 Cytotoxicity assays

Total CTL activity was assessed in an anti-CD3 redirected killing assay using the P815 murine mastocytoma cell line (ATCC TIB-64). Freshly-isolated PBMC, at E:T ratios of 50:1, 25:1, and 12.5:1, were used to assess circulating total CTL activity. AMLR-stimulated effector cells were tested for total CTL activity at ratios of 20:1, 10:1, and 5:1. P815 cells (1×10^6) were labeled for 90 minutes with 100 μ Ci Na₂⁵¹CrO₄ (Amersham Life Sciences, Arlington, IL), washed four times with PBS containing 1% FBS, counted, and resuspended at 1×10^5 /ml. Effector and target cells were incubated in duplicate in 96-well culture plates at a final volume of 300 μ l/well. The anti-CD3 mAb OKT3 (ATCC CRL8001) was added to a final concentration of 1 μ g/ml at the time of the assay. Control wells contained effector and target cells in the absence of anti-CD3. Minimum

and maximum release wells contained target cells in medium or 1N HCl respectively. After 5 hours incubation, 100 μ l cell-free supernatant was removed and then counted in a Wallac 1480 gamma counter. Percent killing was determined by the formula:

$$(\text{Test } ^{51}\text{Cr release} - \text{spontaneous release}) \times 100 / \text{maximum } ^{51}\text{Cr release} - \text{spontaneous release}.$$

Spontaneous release was less than 25% in all assays. Results were considered positive when the percent specific ^{51}Cr release in the test wells was at least 10% above that of the control wells.

HIV-specific CTL activity was assessed using autologous or HLA-matched BLCL infected with the following recombinant vaccinia viruses: vVK1 (*gag/pol*), vDK1 (*gag*), vCF21 (*pol*), vPE16 (*gp160*), vTFnef (*nef*), and vSC8 (E. Coli β -galactosidase, control) (all from the NIH AIDS Research and Reference Reagent Program, Rockville, Maryland). 1×10^6 BLCL were infected with 15 plaque forming units (pfu)/cell overnight and labeled with ^{51}Cr as described above. In most cases E:T ratios were 50:1, 25:1, and 12.5:1. The assay was performed as described above, except the anti-CD3 antibody OKT3 was added to a concentration of 5 $\mu\text{g/ml}$ to duplicate wells containing the high E:T ratio to confirm the role of the TCR in target cell killing. In all cases where HIV-specific CTL activity was reported, lysis was inhibited by OKT3.

2.3.7 T cell repertoire analysis

TCR β chain V gene family expression levels were assessed as in [37] with slight modifications. Briefly, RNA was extracted from bead bound CD8^+ T cells using Trizol

(Gibco BRL, Burlington, ON) and cDNA was synthesized with a first strand cDNA kit (Pharmacia Biotech Inc., Baie d'Urfe, Quebec). The equivalent of 1 μ g RNA was split into 24 polymerase chain reaction (PCR) mixtures, each incorporating 1 of 24 TCR β V gene family-specific primers, a common C β primer and a pair of C α primers for an internal positive control [primers as in 37]. Reactions were run in 20 μ l volumes for 30 cycles under standard conditions, and products separated on 2% agarose gels. Digital image analysis, with "Alphaease" software, was used to express β V band intensities as a fraction of the internal control C α band intensity to compare relative expression levels of individual β V families between samples. TCR β V gene intrafamily junctional length diversity was assessed by separating PCR products on 6% sequencing gels and visualizing individual bands (spectratypes) by silver staining. This yields 6-9 bands per family each differing from the next by 3 base pairs in length [38].

2.4 Results

2.4.1 Subject characteristics

General immunological characteristics of the 13 subjects included in this study together with their HIV virus load measurements at time of study entry are listed in table 1. All subjects had total T cell counts $<500/\mu\text{l}$ on at least two occasions prior to their inclusion in the study. Over the following 12 months, T cell numbers remained relatively stable for all subjects except 028, 069, and 083. These individuals had substantial increases in their T cell numbers and all 3 had T cell counts well above $500/\mu\text{l}$ 12 months from entry into the study. Virus load measurements also remained relatively stable for all subjects except 083, who had steadily decreasing plasma virus levels over this same period. Increasing CD8^+ T cell counts in subjects 028 and 069 were associated with sustained suppression of plasma virus load at undetectable (<400 copies HIV RNA/ml) levels. All of the 13 individuals included in this study received triple combination antiretroviral therapy consisting of either 2 nucleoside analogue reverse transcriptase (RT) inhibitors (NRTIs) with a protease inhibitor (PI), 1 non-nucleoside analogue RT inhibitor (NNRTI) and 1 NRTI with a protease inhibitor or 1 NRTI with 2 PIs. Sustained responses in terms of virus load suppression or remission of lymphopenia did not occur in any of the other 10 individuals despite their receiving equally potent antiretroviral therapy. Subject 029 discontinued all antiretroviral therapy when no substantial reduction in plasma virus load was achieved. No clinical symptoms of opportunistic infections were apparent at times of immunologic testing for any of the individuals.

Table 1. Subjects' Immunologic Characteristics and HIV Plasma Virus Load at Study Entry

Subject ID	T cells/ μl blood	CD4 ⁺ T cells/ μl blood	CD8 ⁺ T cells/ μl blood	% of CD8 ⁺ T cells CD28 ⁺	Log ₁₀ HIV RNA (copies/ml)	†CD8 ⁺ T cell Expansion <i>in vitro</i>	‡Percent redirected lysis of P815 cells	
							Circulating effectors	Cultured effectors
*004	264	8	232	82	5.69	8x	4	36
*005	271	10	230	47	5.1	3x	10	40
*009	203	36	143	65	5.86	3x	1	32
*023	312	21	256	62	5.31	6x	3	28
*028	176	3	141	86	4.48	5x	37	57
*029	442	42	409	57	5.21	5x	7	29
*038	231	0	148	65	5.85	5x	10	40
56	204	20	164	44	5.13	2x	5	90
60	176	0	136	70	4.8	6x	§ND	71
*069	444	52	348	30	2.85	3x	7	63
79	333	64	243	68	2.6	4x	1	34
83	325	84	218	47	5.88	9x	6	65
*091	465	46	328	57	5.88	2x	ND	46

* Subjects with previous AIDS-defining illnesses.

† Approximate *in vitro* expansion over 10 days of stimulation, based on total starting and final CD8⁺ T cell numbers derived from flow cytometry records and cell counts.

‡ Refers to percent anti-CD3 redirected lysis of P815 cells at high E:T ratios; 50:1 for freshly-isolated cells and 20:1 for *in vitro* stimulated cells.

§ ND = not done.

2.4.2 Proliferative and differentiation potential of CD8⁺ T cells in advanced HIV infection

The proliferative potential of CD8⁺ T cells was assessed directly, from the level of expansion following stimulation, and indirectly, from the proportion of CD28⁺ cells in the CD8⁺ T cell population. Results are shown in Table 1. CD8⁺ T cells from all 13 subjects proliferated *in vitro*, with expansion levels ranging from 2x to 9x. This shows that at least some of the CD8⁺ T cells remaining at this advanced stage of infection can still replicate when appropriately stimulated. CD28⁺ cells accounted for the majority of circulating and cultured CD8⁺ T cells in 9 of 13 subjects (table 1 and fig. 1). This is in contrast to a control group of subjects at an earlier stage of infection, matched for viral load measurements but having total circulating T cell counts above 1000/ μ l, where none had >50% CD28⁺ CD8⁺ T cells, χ^2 with Yates correction = 10.82; $p < .005$. In this control group, the percentage of circulating CD8⁺ T cells expressing CD28 ranged from 20% to 45% (mean \pm SD = 35 \pm 7%). We selected this control group of HIV-infected individuals at an earlier stage of infection for comparison of circulating total CTL and inducible anti-HIV CTL, as well as to illustrate effects specific to advanced HIV as opposed to HIV infection per se. Matching for virus load was done to demonstrate that immunological differences did not merely reflect the high circulating plasma virus load common in advanced HIV infection.

Total CTL activity was tested by anti-CD3 redirected lysis of P815 cells. Of 11 subjects tested for total circulating CTL, only one was initially positive (table 1). For 2 individuals who initially tested negative, circulating CTL activity became detectable following significant increases in their T cell counts. In contrast, in the control group

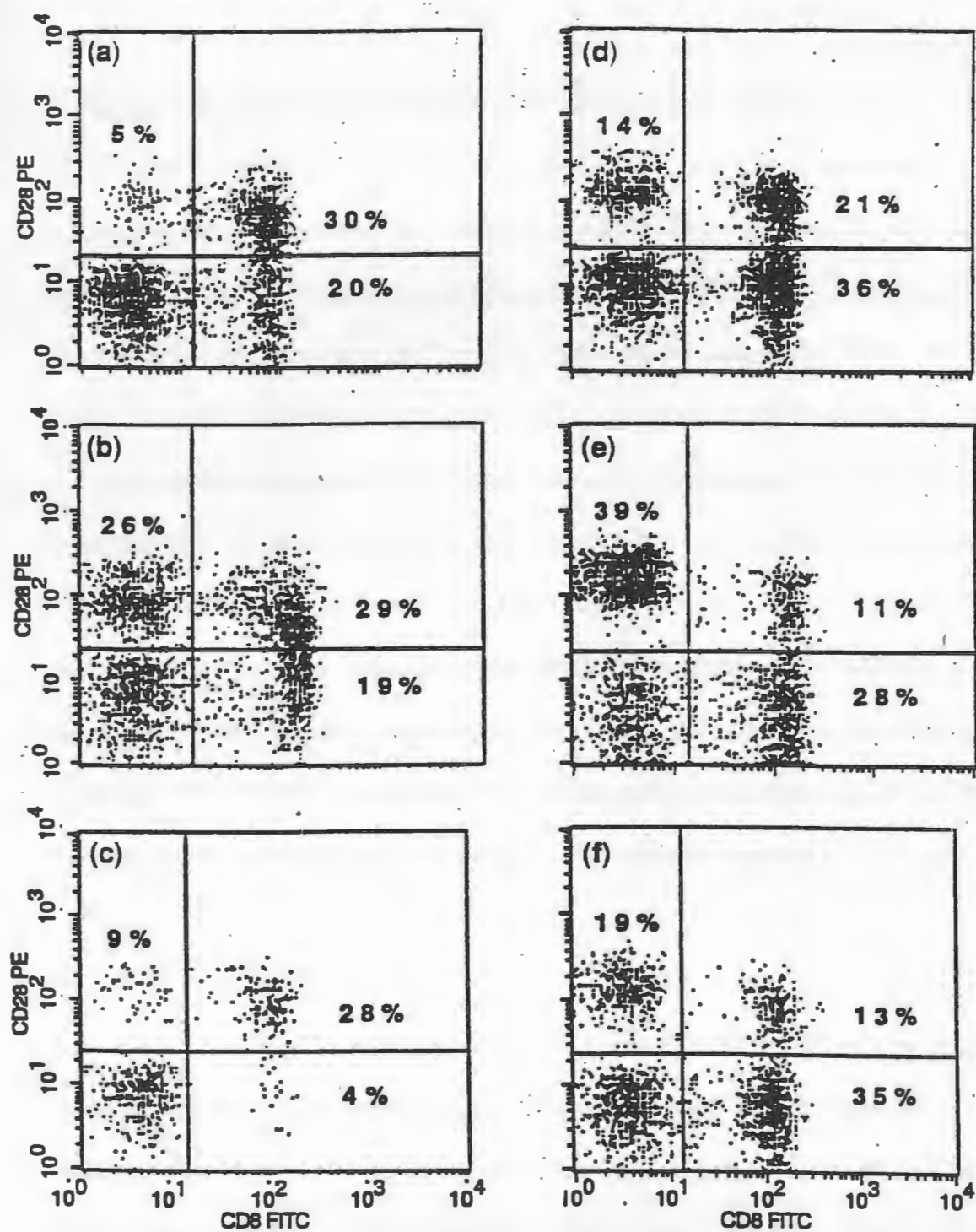


Fig. 1. Representative flow cytometry plots of CD28 expression on circulating peripheral blood CD8⁺ T cells for 3 HIV-infected individuals with advanced HIV infection (a, b and c) and 3 HIV-infected individuals from the control group with >1000 T cell/ml peripheral blood (d, e and f). The percentage of analyzed cells within each quadrant is noted in the plots.

matched for plasma virus load but with >1000 total T cells/ μ l peripheral blood, 13/13 had circulating CTL activity, χ^2 with Yates correction = 16.65; $p < .001$. Total circulating CTL activity at an E:T ratio of 50:1 ranged from 14% to 60% (mean \pm SD = $33 \pm 14\%$) in the control group. This same assay was performed following *in vitro* stimulation, to determine the capacity of the CD8⁺ T cells to differentiate into functional CTL. All subjects in both the advanced-stage group and the control group had demonstrable total CTL activity following *in vitro* stimulation, indicating that functional CTL precursor cells were present in all cases (table 1). At an E:T ratio of 20:1, total stimulated CTL activity in the control group ranged from 31% to 87% (mean \pm SD = $65 \pm 15\%$). These results demonstrate that the CD8⁺ T cell population persisting in advanced HIV infection is mostly CD28⁺ and includes T cells with the capacity to proliferate and differentiate into functional CTL. However, circulating CTL activity appears dependent upon a minimal circulating total T cell number and is usually undetectable in advanced HIV infection.

2.4.3 HIV-specific CTL

Anti-HIV CTL activity after stimulation by AMLR was detected by ⁵¹Cr release assays using autologous or HLA-matched BLCL target cells infected with recombinant HIV/vaccinia viruses. Twelve subjects were tested for anti-HIV CTL activity against *gag*, *pol*, *env* and *nef*, with 6 testing positive against at least 1 antigen. In the control group described above, 11/11 showed anti-HIV CTL activity against at least 1 antigen, χ^2 with Yates correction = 5.09; $p < .025$ (data not shown). Figure 2 shows the cytotoxicity results for the 6 subjects with detectable anti-HIV CTL activity. Anti-HIV CTL activity

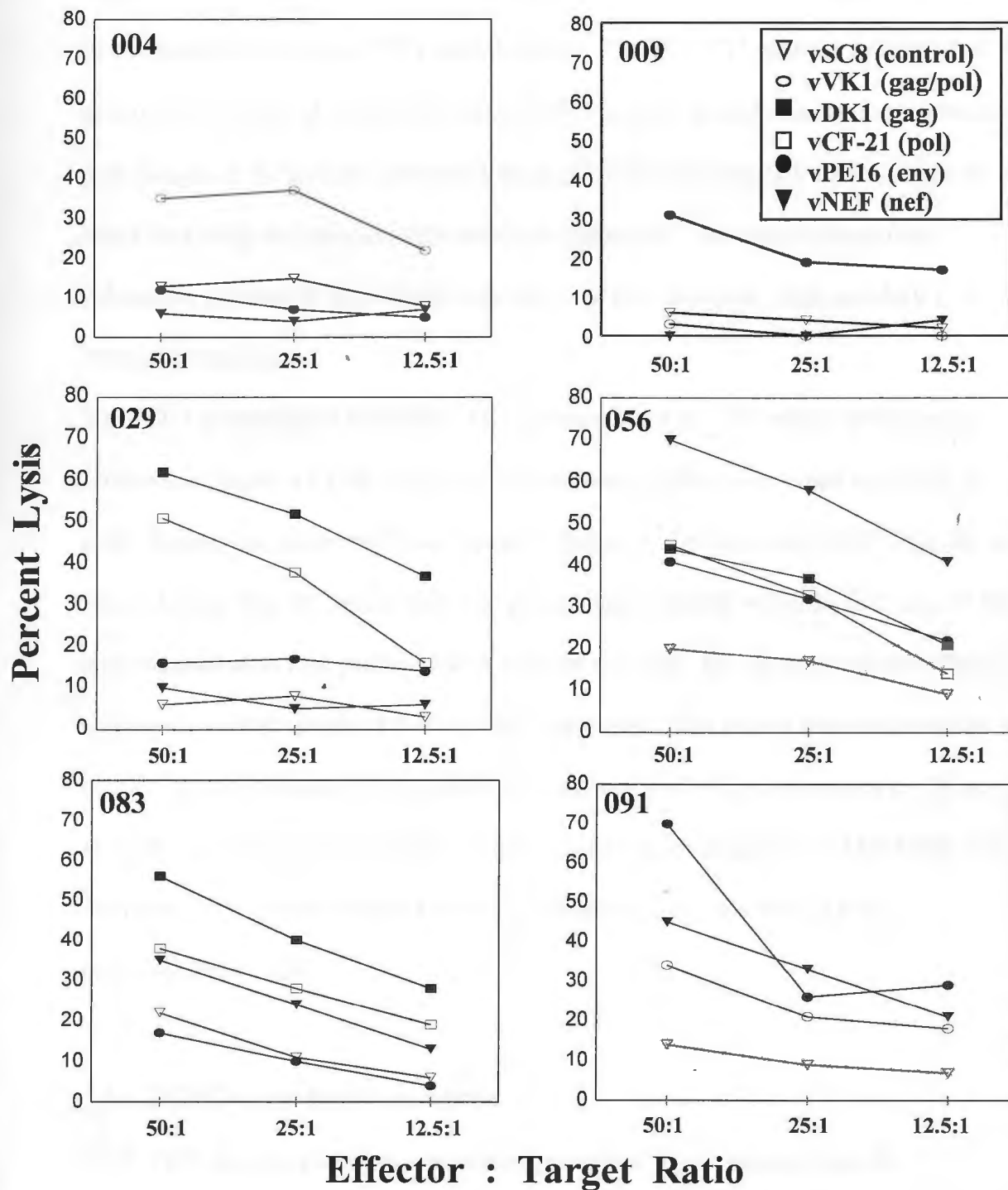


Figure 2. Anti-HIV CTL activity of 6 subjects with advanced HIV infection. The target cell legend depicted in the second panel applies to all graphs. The number in the upper left hand corner of each graph corresponds to subject numbers in Table 1.

ranged from narrow (1 antigen) and weak (<25% specific lysis), to broad (>3 determinants) and strong (>50% specific lysis). Anti-HIV CTL activity in the control group of HIV-infected individuals with >1000 T cells/ μ l ranged over the same breadth and strength as the activity detected in the group with advanced HIV infection. In all cases the killing was blocked with anti-CD3 antibodies. This demonstrates that a substantial fraction of individuals with advanced HIV infection retain anti-HIV CTL memory responses.

The CD28 phenotype of anti-HIV CTL was determined by ^{51}Cr release assays using effectors depleted of CD28⁺ cells, and in some cases, effectors enriched for CD28⁻ T cells. Results for subject (029) are shown in Figure 3. Depletion of CD28⁺ cells did not reduce killing (fig. 3a), while there was an increase in activity when the E:T ratio of 50:1 was re-established with purified CD28⁻ effector cells (fig. 3b). In this case, the killing activity was clearly confined to the CD28⁻ population. This testing was performed for 4 of the 6 subjects showing HIV-specific CTL activity (009, 029, 056, and 083). Of the 10 anti-HIV CTL responses assessed, 8 were mediated predominantly by CD28⁻CD8⁺ CTL. Therefore, when present at this late stage of infection, HIV-specific CTL are predominantly CD28⁻.

2.4.4 TCR β V gene family expression

CD8⁺ TCR repertoire analysis was performed on 9 of the 13 subjects and, for comparison, on 6 uninfected controls. Six uninfected individuals were chosen as a control group for the TCR repertoire analysis as perturbations of the TCR repertoire have

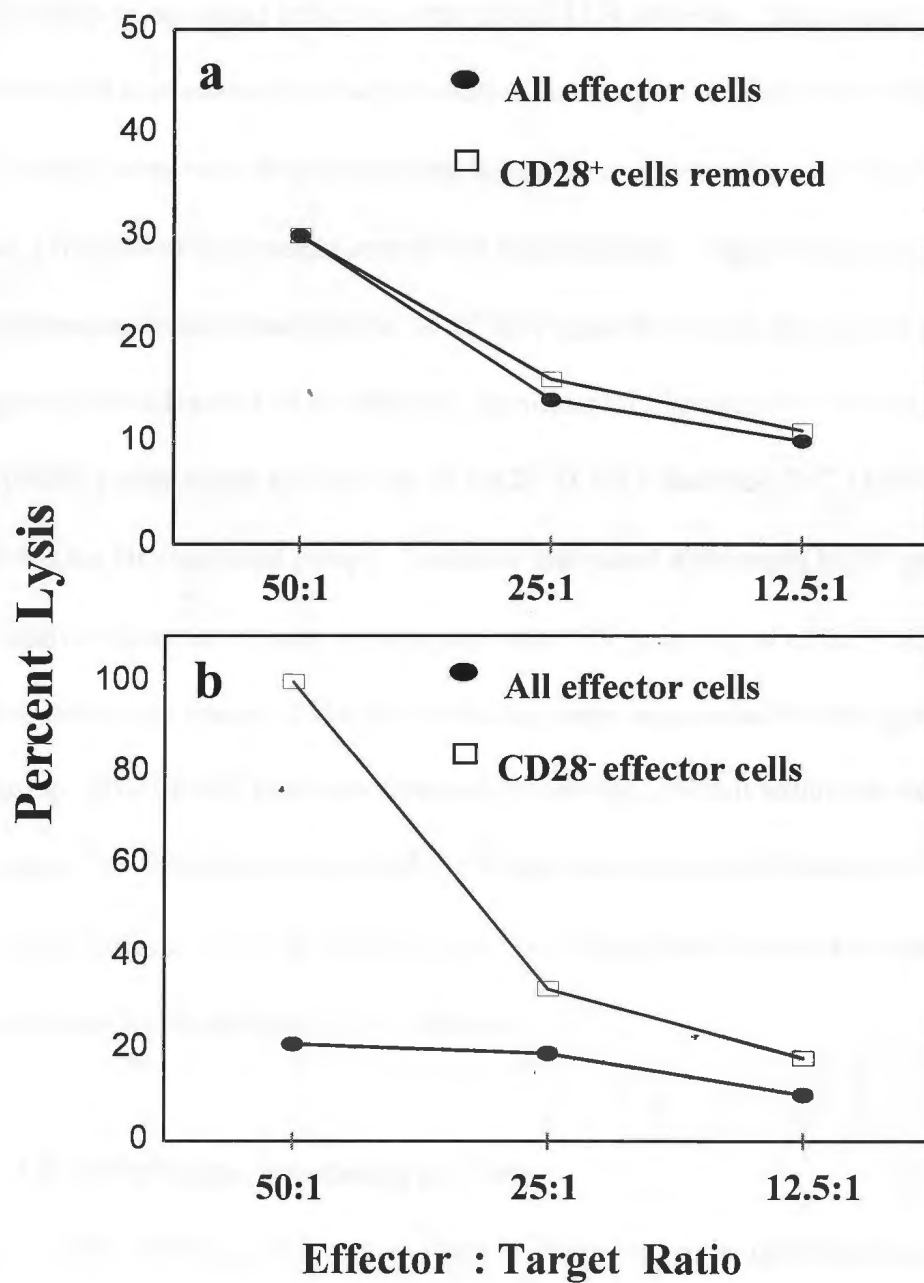


Figure 3. Effects of depletion of CD28⁺ T cells (panel a) or enrichment of CD28⁻ T cells (panel b) on the anti-HIV CTL activity of subject 029. Target cells were HLA Bw62-matched BLCL infected with vVK1, to detect Bw62-restricted anti-HIV CTL against either *gag* or *pol*.

been reported at different stages of HIV infection and we wanted to compare TCR diversity in advanced infection with normal TCR diversity. Semi-quantitative RT-PCR was used to examine the relative levels of transcripts for 24 different TCR β V families. Relative expression levels were determined by expressing the specific β V band intensity as a fraction of the internal control C α band intensity. Figure 4 compares mean relative expression levels of each of the 24 TCR β V gene families in the control group to the group with advanced HIV infection. Significant differences between the 2 groups ($p < 0.05$) were found for only one of the 24 TCR β V families; β V2 (12.0% for controls vs 8.6% for HIV-infected group). To assess individual differences in β V gene family relative expression levels, we compared each β V gene family of the 9 HIV-infected subjects to the mean \pm 2 standard deviation range established for the uninfected control group. Of 210 total reactions analyzed in this way, 201 fell within the established normal range. The 9 deviations involved 5 different subjects and 6 different β V families. These results indicate that CD8⁺ TCR β V gene family expression levels are essentially normal in individuals with advanced HIV infection.

2.4.5 TCR β V gene intrafamily diversity

For a more detailed examination of the TCR repertoire, we performed junctional length diversity analysis within different TCR β V families. PCR amplification with β V gene family and C β specific primer pairs spanning the VDJ junction and separation of the products on sequencing gels reveals, on average, 6 to 9 different length polymorphisms in a normally diverse T cell repertoire [38]. We assessed junctional length diversity within

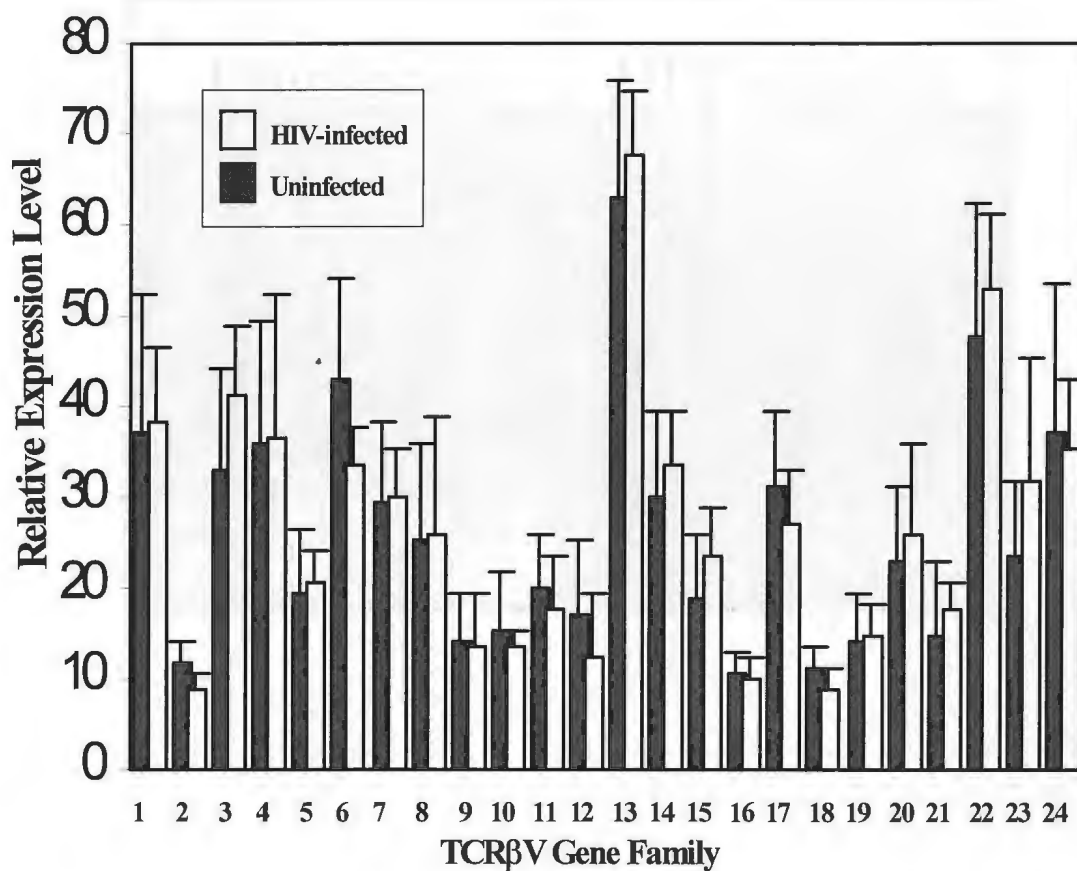


Figure 4. Mean relative TCRβV family expression levels (\pm standard deviation) of 9 HIV-infected individuals with advanced HIV infection (light bars) compared to 6 uninfected controls (dark bars). The intensity of individual βV family bands was expressed as a percentage of the internal control αC band intensity to normalize relative expression levels.

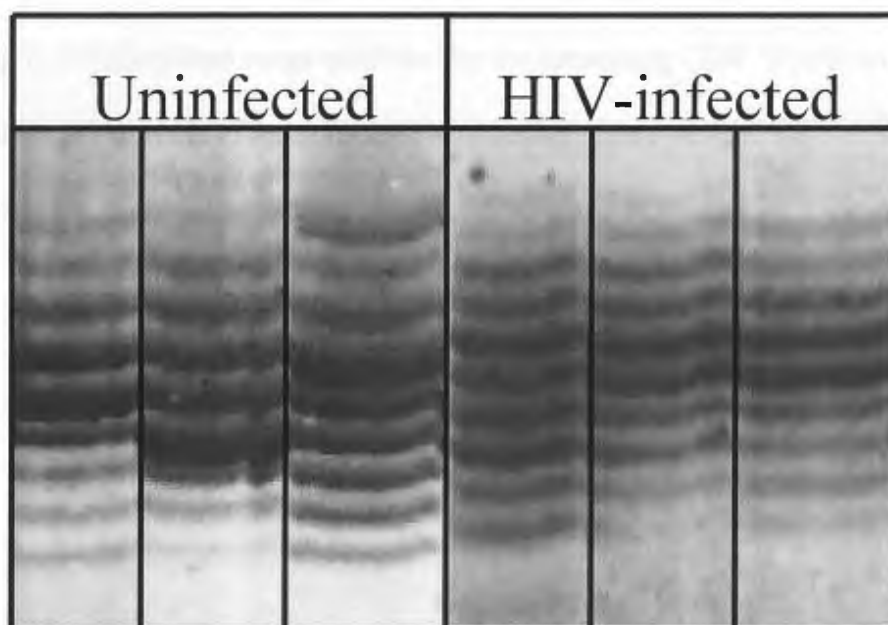


Figure 5. TCR β V22 gene intrafamily junctional length diversity analysis comparing 3 uninfected controls and 3 individuals with advanced HIV infection. Individual β V22 gene family PCR products were separated on 6% polyacrylamide sequencing gels and visualized by silver staining.

14 β V families, and figure 5 shows results for one family (β V22) which are representative of our overall assessment. The number of individual bands visible was counted to assess overall diversity and no significant differences between the control and test group were found when the average number of bands in each family was compared. Stimulated cells were used to examine the diversity of the TCR β V repertoire in the HIV-infected individuals and, although this might allow detection of rare spectratypes, it can not increase the overall diversity of the repertoire. All spectratypes observed were within the length polymorphism range exhibited by the circulating CD8⁺ T cells analyzed from the uninfected controls. From this data we conclude that overall CD8⁺ TCR repertoire diversity is largely maintained in advanced HIV infection.

2.5 Discussion

In this study we investigated functional and genetic characteristics of the CD8⁺ T cell population in individuals with advanced HIV infection. The majority of CD8⁺ T cells persisting in this group expressed the T cell co-stimulatory molecule CD28, which has been linked with telomere length and replicative capacity [21-23]. CD8⁺ T cells from all individuals proliferated *in vitro* and differentiated into functional CTL. Both TCRβV family expression levels and TCRβV gene intrafamily diversity levels largely fell within the same range as exhibited by uninfected controls. Together, these results indicate that from a general perspective, the functional and molecular genetic integrity of the CD8⁺ population is maintained in advanced HIV infection.

We also studied HIV-specific reactivity in the CD8⁺ T cell population in advanced HIV infection and found that half of the individuals tested retained inducible anti-HIV CTL activity against at least one of HIV *gag*, *pol*, *env* or *nef*. Because HIV-specific CTL are thought to be important in controlling viral replication, the persistence of vigorous and, in some cases, diverse virus-specific memory CTL responses in subjects with advanced disease is somewhat paradoxical. The lack of detectable circulating CTL activity in these individuals indicates that no active CTL response is ongoing, despite high levels of viremia. One explanation for this is that CTL escape mutants have emerged, rendering the HIV-specific CTL detected at this stage ineffectual. The rapid rate of HIV replication combined with the high error rate of the RT enzyme produces a high mutation rate for HIV [39, 40]. Therefore, it is reasonable to speculate that mutations in CTL epitopes serve as a mechanism of immune escape. Although this has been questioned for HIV

infection, there is now considerable evidence showing that escape of HIV from CTL recognition plays a role in viral persistence [1, 15, 41-44]. This may be especially true where an oligoclonal HIV-specific CTL response involving recognition of only a single variable epitope develops. In these cases, HIV can easily escape and very rapid disease progression has been observed [44]. Emergence of CTL escape mutants has been demonstrated in primary infection [1, 42] and, in two cases, delayed immune escape was observed after 9-12 years of infection [15]. Together these reports clearly support the hypothesis that sequence variation serves as a mechanism of HIV escape from CTL.

However, it is unlikely that sequence variation alone accounts for the persistence of strong HIV-specific memory CTL responses in subjects with advanced disease. Two of our subjects (056 and 091) had a diverse HIV-specific CTL response and simultaneous complementary mutations of multiple epitopes would be required for complete CTL escape. In cases such as this, it may be more likely that another mechanism, such as anergy or deprivation of CD4⁺ T cell help, renders the anti-HIV CTL ineffective. Since anergic cells usually regain responsiveness after a period of growth in IL-2 (45), our stimulation procedure would overcome this, but we have never observed much cell growth at all, let alone development of CTL function simply by exposing T cells from individuals at this stage of infection to IL-2.

It is also possible that the HIV-specific CTL present at this stage of infection are CTL that have entered a state of replicative senescence on the verge of clonal exhaustion, and are ineffective *in vivo*. Our finding that anti-HIV CTL were predominantly CD28⁻ lends some credence to this theory, however, the percentage of CD8⁺ T cells expressing CD28 was comparable in fresh and stimulated cell cultures. This indicates that either CD28⁻

cells replicate as efficiently as CD28⁺ cells, which would mean they were not all senescent cells, or that a proportion of the CD28⁺ population loses CD28 expression during culture *in vitro*. If so, the anti-HIV CTL memory cells could be predominantly CD28⁺. For several individuals, we detected anti-HIV CTL of the same specificity on multiple occasions over a 12 month period in spite of a consistently high (>5log₁₀/ml) plasma virus load, arguing against these cells being on the verge of antigen-driven clonal exhaustion.

Six other individuals with advanced infection showed no detectable anti-HIV CTL activity on initial testing. It is possible that these individuals did have HIV-specific CTL present at very low precursor frequencies or that were directed toward epitopes not expressed by the recombinant vectors used, but the lack of detectable activity in 50% of the individuals tested suggests anti-HIV CTL are clonally exhausted in some cases of advanced HIV infection. Exhaustion of anti-viral CTL has been shown to occur in mice infected with rapidly-replicating strains of lymphocytic choriomeningitis virus (LCMV) [46]. A similar situation could occur in HIV-infected individuals, where prolonged high-level stimulation of activated effector CTL could produce clonal exhaustion by pushing cells beyond their replicative lifespan, [19, 47]. Studies have shown that CD8⁺ T cells of HIV-infected individuals have significant telomere shortening, indicating limited replicative reserve and the potential for clonal exhaustion [21, 22]. There is also evidence that rapid exhaustion of HIV-specific CTL clones can occur during primary infection, with emergence of new dominant clones [27]. If exhaustion were to occur in the latter stages of disease, when T cell numbers are severely compromised, it may be impossible to generate new responses, resulting in a lack of inducible anti-HIV CTL

activity. However, in two cases where HIV-specific CTL were initially undetectable, a specific response became apparent following remission of T cell lymphopenia, suggesting that reconstitution of HIV-specific CTL responses may still be possible even at this late-stage of disease. Whether this inducible activity translates into an active, effective CTL response *in vivo* remains to be seen, but the fact that these individuals now show detectable circulating CTL activity as well is a positive sign.

In summary, our results suggest that in advanced infection, HIV-specific CTL activity is selectively compromised while the functional and genetic integrity of the CD8⁺ T cell population as a whole remains intact. A substantial fraction of individuals with advanced HIV infection retain anti-HIV CTL memory cells, however, these cells may be incapable of differentiating into effector CTL *in vivo* and further work is necessary to determine if these CTL recognize the contemporaneous endogenous HIV strains present. For other individuals, HIV-specific CTL are undetectable at this stage of disease. Therefore, multiple mechanisms appear to contribute to the loss of CTL activity in progressive HIV infection. However, the functional and genetic integrity of the CD8⁺ population as a whole is maintained, and since half of the HIV-infected individuals at this stage retain anti-HIV memory cells and another third regained HIV-specific CTL coincident with treatment-induced increases in T cell numbers, CTL-based immunotherapies for HIV or other pathogens remain plausible even in the advanced stages of HIV infection.

2.6 Acknowledgments

The authors would like to thank the United States National Institute of Allergy and Infectious Diseases (NIAID) AIDS reference reagent program for providing recombinant vaccinia viruses and recombinant human interleukin-2. We are also grateful to the HIV-infected individuals who participated in this study of immune function in advanced HIV infection.

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CHAPTER 3

Immune Reconstitution and Viral Stimulation are Required to Restore HIV-specific CD8 T Cell Responses Following Advanced Infection

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3.1 ABSTRACT

The extent to which highly active antiretroviral therapy (HAART) restores human immunodeficiency virus (HIV)-specific immunity in advanced infection is unknown. Therefore, we studied how effective therapy affected HIV-specific CD8⁺ T cell responses in 4 individuals who had progressed to advanced infection. CD8⁺ T cell responses were assessed by cytotoxicity and interferon-gamma (IFN- γ) production. Proliferative CD4⁺ T cell responses against HIV, *Candida* and mitogen were measured by ³H-thymidine incorporation. Substantial immune reconstitution indicated by increased CD4⁺ and CD8⁺ T cell numbers followed suppression of viral replication. This was associated with emergence of HIV-specific cytotoxic T lymphocytes (CTL), but only concurrent with detectable viral replication. Emergent anti-HIV CTL were similar to those at earlier stages of infection in terms of their specificity, function and CD28 phenotype. However, they were very short-lived in the absence of detectable HIV replication. Antigen-specific CD4⁺ T cell responses remained severely compromised. Thus, effective antiretroviral therapy restores the capacity for HIV-specific CTL responses after advanced infection. However, the transient nature of these responses suggests failure to generate stable long-lived memory cells in the absence of HIV-specific helper T cell responses.

Keywords: HIV, HAART, cytotoxic T lymphocytes, Immune reconstitution

3.2 INTRODUCTION

The central antiviral role of CD8⁺ T cells in simian immunodeficiency virus (SIV) infection has been clearly demonstrated (1,2) and many studies support a similar role for cytotoxic T lymphocytes (CTL) in human immunodeficiency virus (HIV) infection (reviewed in 3,4). The anti-HIV CTL response is remarkably strong and diverse, as HIV-infected individuals often have high frequencies of CTL against many HIV epitopes (5,6). Untreated HIV infection almost invariably progresses to acquired immune deficiency syndrome (AIDS) as the levels and function of HIV-specific CTL diminish (7,8). Several theories addressing the failure of CTL to durably suppress HIV replication include loss of HIV-specific CD4⁺ helper T cells (9,10) and accumulation of CD8⁺CD28⁻ T cells with limited proliferative potential (11,12). Since virus and host co-exist for extended periods with many cumulative detrimental effects on the immune system, it is not surprising that multiple mechanisms might drive the ultimate failure of HIV-specific CTL. Whatever underlies this failure, it is important to determine if a 'point of no return' exists, beyond which HIV-specific CD8⁺ T cell responses are precluded, or if even following advanced HIV infection, immune reconstitution enables specific immune responses against HIV.

Highly active antiretroviral therapy (HAART) can efficiently suppress viral replication, increase T cell numbers and promote immunity against certain opportunistic pathogens, even when initiated after advanced HIV infection (13-15). However, the degree of HIV-specific immune reconstitution that can occur at this late stage is unknown. To address this issue, we followed HIV-infected individuals who had reached advanced infection

before responding to therapy, since any intractable defects imposed upon HIV-specific immunity over progressive HIV infection should be especially evident in this group. We previously characterized CD8⁺ T cell responses in a group of HIV-infected individuals with advanced infection (7). Four of these individuals subsequently responded to HAART with viral replication suppressed to undetectable levels and increases in their T cell counts. In this report, we describe associated changes in their HIV-specific CTL responses as well as their CD4⁺ T cell responses against Candida and HIV p24.

For 2 individuals without anti-HIV CTL, HIV-specific CTL emerged following reconstitution of T cell numbers, but only transiently, in parallel with breakthroughs in viral replication. A third subject had undetectable HIV-specific CTL and viral load at all time points. The fourth subject retained diverse anti-HIV CTL while virus load remained detectable, even when T cell numbers were depressed, but rapidly lost HIV-specific CTL once virus replication became undetectable. Although these findings demonstrate that immune reconstitution enables HIV-specific CTL responses following advanced infection, the transient nature of these responses also illustrates the residual impact of cumulative immune defects imposed over the course of progressive HIV infection.

3.3 MATERIALS AND METHODS

3.3.1 *Study subjects*

Study participants were recruited through the Infectious Disease Clinic of the St. John's General Hospital, St. John's, NL, Canada. All had progressed to advanced HIV infection, defined by a total T cell count of $<500/\mu\text{l}$ on consecutive clinic visits. At each visit clinical evaluation was performed, plasma HIV RNA was measured using Amplicor HIV-1 MonitorTM kits (Roche Diagnostic Systems Inc., Mississauga, Ontario, Canada) and peripheral blood lymphocyte (PBL) subsets were assessed by flow cytometry. Ethical approval was obtained from the Memorial University Faculty of Medicine Human Investigation Committee and all participants provided informed consent.

3.3.2 *Sample preparation*

Peripheral blood mononuclear cells (PBMC) were isolated from acid-citrate dextrose (ACD)-preserved fresh whole blood by density gradient centrifugation with Ficoll-paque lymphocyte separation medium (Pharmacia Chemicals, Dorval, Quebec, Canada). PBMC were washed twice in phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS; Gibco, Grand Island, New York, USA), resuspended in lymphocyte medium (RPMI 1640 with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2×10^{-5} M 2-mercaptoethanol; all from Gibco) and counted.

Human leukocyte histocompatibility antigen (HLA) class I typing and generation of B lymphoblastoid cell lines (BLCL) were carried out as previously described (7).

3.3.3 Cytotoxicity assays

To activate anti-HIV CTL, approximately 1×10^7 PBMC were stimulated by an autologous mixed lymphocyte reaction (AMLR), as previously described (7). HIV-specific CTL activity was assessed against autologous or HLA-matched BLCL targets infected with the following recombinant vaccinia viruses: vVK1 (*gag/pol*), vDK1 (*gag*), vCF21 (*pol*), vPE16 (*gp160*), vTF*nef* (*nef*), and vSC8 (E. Coli β -galactosidase, control) (all from the NIH AIDS Research and Reference Reagent Program, Rockville, Maryland). Target cells were infected with 15 plaque forming units (pfu)/cell of the desired virus overnight, labeled for 90 minutes with 100 μ Ci $\text{Na}_2^{51}\text{CrO}_4$ (Amersham Life Sciences, Arlington, IL), washed 4 times with PBS containing 1% FBS, counted, and resuspended in lymphocyte medium. Effector and target cells (5000 cells/well) were incubated in duplicate in 96-well culture plates in a final volume of 300 μ l/well at effector:target (E:T) ratios of 50:1, 25:1, and 12.5:1. Minimum and maximum release wells contained targets in medium alone or 1N HCl respectively. After 5 hours incubation, 100 μ l supernatant was removed and counted in a Wallac 1480 gamma counter. Percent killing was determined by the formula:

$$(\text{Test } ^{51}\text{Cr release} - \text{spontaneous release}) \times 100 / \text{maximum } ^{51}\text{Cr release} - \text{spontaneous release}.$$

Spontaneous release was always <25% and results were considered positive when percent specific ^{51}Cr release in the test wells was $\geq 10\%$ above control wells.

Peptide-sensitized targets were used to delineate epitopes recognized by anti-HIV CTL. Synthetic 20-mers at 40 $\mu\text{g}/\text{ml}$ were incubated with ^{51}Cr -labeled target cells (5000/well) for 2 hours in 110 μl lymphocyte medium. Effectors were then added at a 50:1 E:T ratio. Control wells contained effectors and targets without peptides. The volume of each well was adjusted to 300 μl and the 5 hour assay carried out as described. Overlapping *gag* (NIH AIDS Research and Reference Reagent Program) and *nef* (AIDS Reagent Project, Hertfordshire, England) peptides were tested.

3.3.4 Lymphocyte separations

$\text{CD}28^+$ lymphocytes were removed from effector populations immediately before CTL assays as previously described (7). To culture purified $\text{CD}28$ -positive and negative $\text{CD}8^+$ T cells, $\text{CD}4^+$ lymphocytes were depleted from PBMC using magnetic beads coated with anti-human $\text{CD}4$ (DynaI Inc., Great Neck, NY, USA) at a 10:1 bead-to-target cell ratio prior to $\text{CD}28^+$ T cell depletion as in (7). The resulting $\text{CD}4^-\text{CD}28^+$ (beaded) and $\text{CD}4^-\text{CD}28^-$ (unbound) populations were stimulated by AMLR, with irradiation of the PHA-activated PBMC (3000 rads) before co-culture. Five days later, beads were removed from the $\text{CD}4^-\text{CD}28^+$ cell cultures by vigorous pipetting and magnetic removal of released beads. The beads were then washed twice by vigorous pipetting with PBS with 5 mM EDTA and 0.1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, Missouri, USA). The unbound cells were pooled, centrifuged and recultured at $1 \times 10^6/\text{ml}$

in lymphocyte medium with 5 U/ml recombinant human interleukin-2 (IL-2; Hoffmann La Roche, Nutley, NJ, USA).

3.3.5 *Flow cytometry*

To determine the proportion of CD8⁺ T cells expressing CD28, PBMC were washed once in PBS containing 5 mM EDTA and 0.1% BSA and incubated for 20 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (Dako Diagnostics, Mississauga, Ontario), phycoerythrin (PE)-conjugated anti-CD28 and peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 (Becton Dickinson). Samples were then washed, resuspended in 0.5% paraformaldehyde in PBS and analyzed on a FACStar^{Plus} (Becton Dickinson). For intracellular staining, effector cells were incubated for 5 hours at 500,000 cells per well in 1.0 ml medium in 24-well culture plates at 5:1 ratio with autologous BLCL infected with recombinant HIV/vaccinia viruses. Ten µg/ml Brefeldin A (Sigma Chemical Co.) was added for the final 4 hours. After incubation, cells were washed once and incubated for 20 minutes at 4°C with PerCP-conjugated anti-CD8 (Becton Dickinson). Cells were then washed, fixed with 2% paraformaldehyde for 15 minutes at 4°C, washed again and permeabilized with 0.2% saponin (Sigma Chemical Co.) in wash buffer, for 10 minutes at 4°C. The cells were washed once again and incubated for 20 minutes at 4°C with FITC-conjugated anti-interferon gamma (IFN-γ) (Caltag Laboratories, Burlingame, California), or FITC-conjugated isotype-matched controls. Samples were washed again, resuspended in 1% paraformaldehyde in PBS and analyzed on a FACStar^{Plus} analyzer (Becton Dickinson).

3.3.6 Proliferation assays

Lymphocyte proliferation assays were carried out by resuspending PBMC in lymphocyte medium with 10% human AB serum (ICN Biomedicals Inc., Aurora, Ohio) replacing FBS. Cells were incubated at 100,000/well in triplicate in 200 μ l in 96-well culture plates. Negative control wells included 2 μ g/ml β -galactosidase (VWR Canlab, Mississauga, ON) or medium alone, while positive control wells contained 5 μ g/ml purified phytohemagglutinin (PHA-P; Wellmark Diagnostics, Guelph, Ontario, Canada). Test wells contained 2 μ g/ml *Candida albicans* (Greer Laboratories Inc., Lenoir, NC) or HIV p24 (Virogen, Watertown, MA). After 5 days, wells were pulsed with 1 μ Ci 3 H-thymidine (Perkin Elmer Life Sciences Inc., Boston, MA) and 16 hours later were harvested onto filter mats using a Tomtec Harvester96 and counted on a Packard TopCount beta counter. Stimulation indices were determined by dividing 3 H-thymidine incorporation in test wells by that of negative control wells.

3.4 RESULTS

3.4.1 Subject characteristics

Table 1 shows age, gender, duration of follow-up, and early and late CD4⁺ and CD8⁺ T cell counts for the individuals studied. All were initially considered in advanced HIV infection with total T cell counts <500/ μ l peripheral blood. Two had previous AIDS defining illnesses (cytomegalovirus retinitis; 028, mycobacterium avium complex; 069). With varying patterns, control of viral replication and at least partial restoration of T cell counts occurred for all subjects. Changes in T cell counts and HIV virus load over time are shown in figure 1a-d. All subjects received HAART consisting of a protease inhibitor (PI) and at least 2 nucleoside analogue reverse transcriptase inhibitors.

3.4.2 Changes in HIV-specific CTL Activity

Results of HIV-specific CTL assays are indicated in figure 1a-d by plus or minus signs directly below the x-axes; pluses indicate a positive response against at least one HIV antigen tested (*gag*, *pol*, *env*, and *nef*), while minus signs indicate no detectable HIV-specific CTL activity at that time.

While T cell counts remained very low, only subject 083 had detectable anti-HIV CTL. Diverse activity against *gag*, *pol*, and *nef*, was detected on four occasions over the first year of study. Antiretroviral treatment increased T cell numbers over this period, but only partially suppressed virus replication. Once full control of virus replication

Table 1. General Characteristics of HAART-related Immune Reconstitution in This

Study									
Subject ID	Age	Sex	^a Months of follow-up	CD4 ⁺ T cells Per μ l blood		CD8 ⁺ T cells per μ l blood		%CD8 ⁺ CD28 ⁺ T cells	
				^b Early	Late	Early	Late	Early	Late
028	32	M	53	4	247	94	1143	75	42
069	46	M	51	46	184	266	510	28	45
079	30	F	51	48	348	226	480	75	84
083	34	M	38	121	334	347	574	44	48

^aMonths of follow-up refers to the total length of time the individual has been under observation since enrolling in study.

^bEarly and late CD4⁺ and CD8⁺ T cell counts/ μ l peripheral blood are averages of the first 2 (early) and last 2 (late) such measures available for each participant.

occurred, HIV-specific CTL became undetectable (figure 1a). Subject 079 had no detectable HIV-specific CTL or viral load at any time. HAART initiated prior to the start of this study had controlled virus replication and while continued therapy increased T cell counts, HIV-specific CTL never became detectable (figure 1b).

Subjects 028 and 069 showed similar patterns of HIV-specific CTL activity, T cell reconstitution and viral replication. Both initially had very low T cell counts and detectable virus replication, but no HIV-specific CTL (figure 1c and 1d). Over several months of effective therapy, T cell counts increased and plasma virus load became undetectable. HIV-specific CTL were detected subsequent to partial T cell restoration only when breakthroughs in viral replication occurred. This CTL activity rapidly disappeared as control of viral replication was reestablished. Subject 028 experienced a brief breakthrough in virus replication concurrent with HIV-specific CTL activity that persisted for 3 months (figure 1c). Subject 069 experienced a more sustained breakthrough in viral replication. Anti-HIV CTL against *gag* and *nef* were detected shortly before the breakthrough and when virus replication persisted, the response broadened to include *pol*. Shortly after this, virus load again became undetectable and all HIV-specific CTL disappeared (figure 1d). These results indicate that following advanced HIV infection, development of new CTL responses depends on immune reconstitution, and that persistent antigenic stimulation is required to maintain HIV-specific CTL activity.

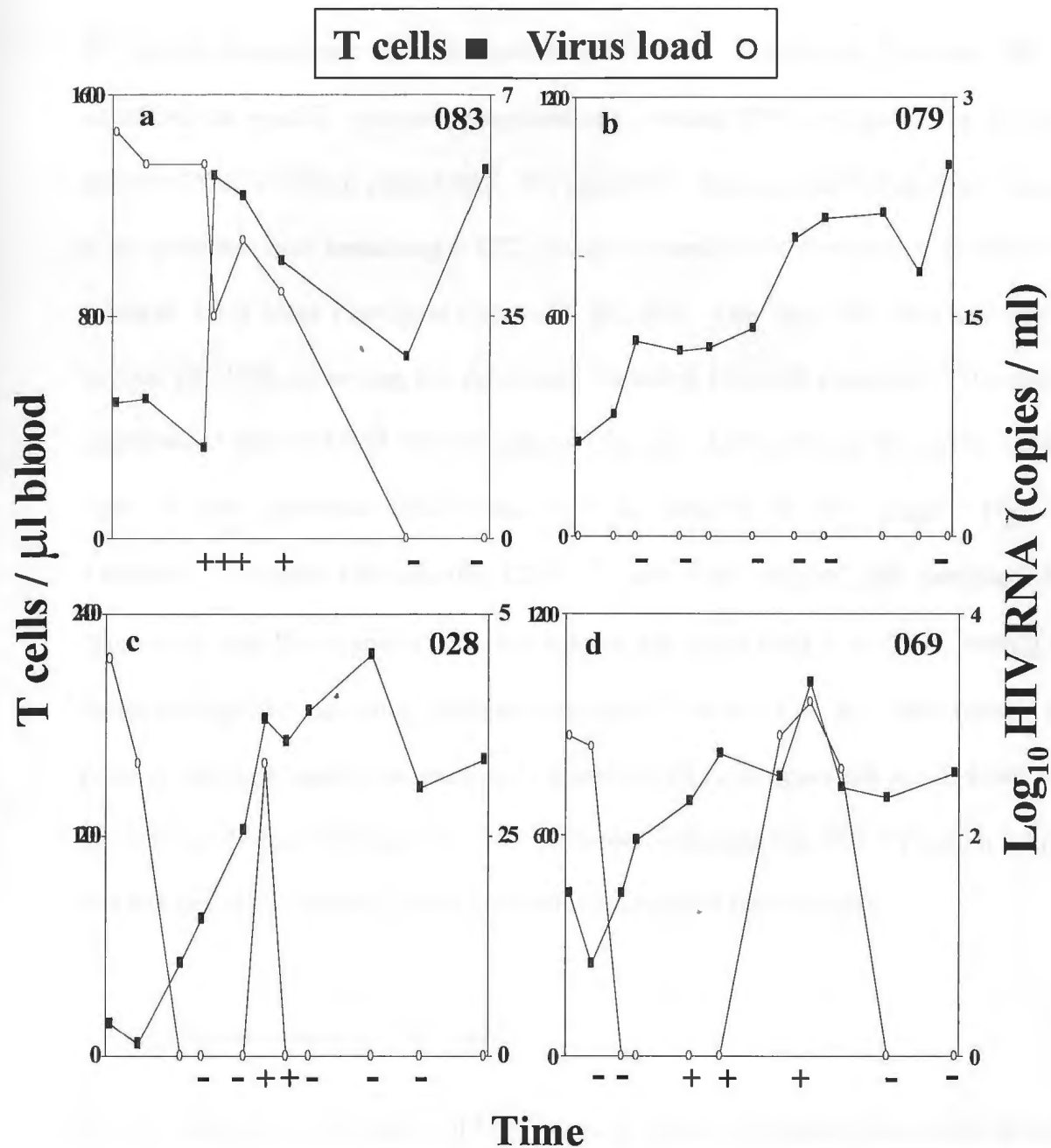


Figure 1. Changes in T cell counts and plasma HIV RNA levels for 4 individuals with severe lymphopenia prior to responding to antiretroviral therapy. Pluses and minuses directly below the x-axis indicate time points at which HIV-specific CTL activity was present (+) or absent (-). On the y-axis, a viral load of '0' indicates viral replication below detection limits of the assay, which changed from log_{10} 2.6 to log_{10} 1.7 copies/ml during the course of study. Subjects are identified in the upper right corner of each graph and the duration of study for each individual is provided in Table 1.

3.4.3 Characterization of emergent anti-HIV CTL

To further characterize the emergent anti-HIV CTL of subjects 028 and 069, we identified the specific epitopes recognized and assessed IFN- γ production by the HIV-specific CD8⁺ T cells of subject 069. We tested CTL from subject 069 against 7 *gag* and 9 *nef* peptides, each containing a CTL epitope presented in association with one of the subjects' HLA class I antigens (A3, A28; B7, B8). One *gag* (321-340) and one *nef* peptide (81-100) containing the previously identified HLA-B8 restricted CTL epitopes *gag* 329-337 and *nef* 87-95 were recognized (fig. 2a). Approximately 4% of the subjects' CD8⁺ T cells produced IFN- γ specifically in response to HIV *gag/pol* (fig. 2b). Therefore, emergent HIV-specific CD8⁺ T cells from subject 069 mediated both cytotoxicity and IFN- γ production. For subject 028 (HLA class I: A2; B35, Bw62) five 9-mer *pol* peptides containing epitopes presented by HLA-A2 or -B35 were tested. One positive response against the previously identified HLA-A2 restricted *pol* 334-342 CTL epitope was detected (data not shown). Therefore, emergent anti-HIV CTL from subjects 028 and 069 were directed against commonly-recognized HIV epitopes.

3.4.4 CD28 phenotype of CD8⁺ T cells

Several studies at earlier stages of HIV infection reported increased proportions of CD8⁺ T cells expressing CD28 with effective suppression of HIV replication (16,17). In contrast to the earlier stages of HIV infection, we found high proportions of CD28⁺CD8⁺ T cells in advanced infection (7). Therefore, we wanted to see what happened to this T cell subset with effective viral suppression following advanced infection. Table 1 shows

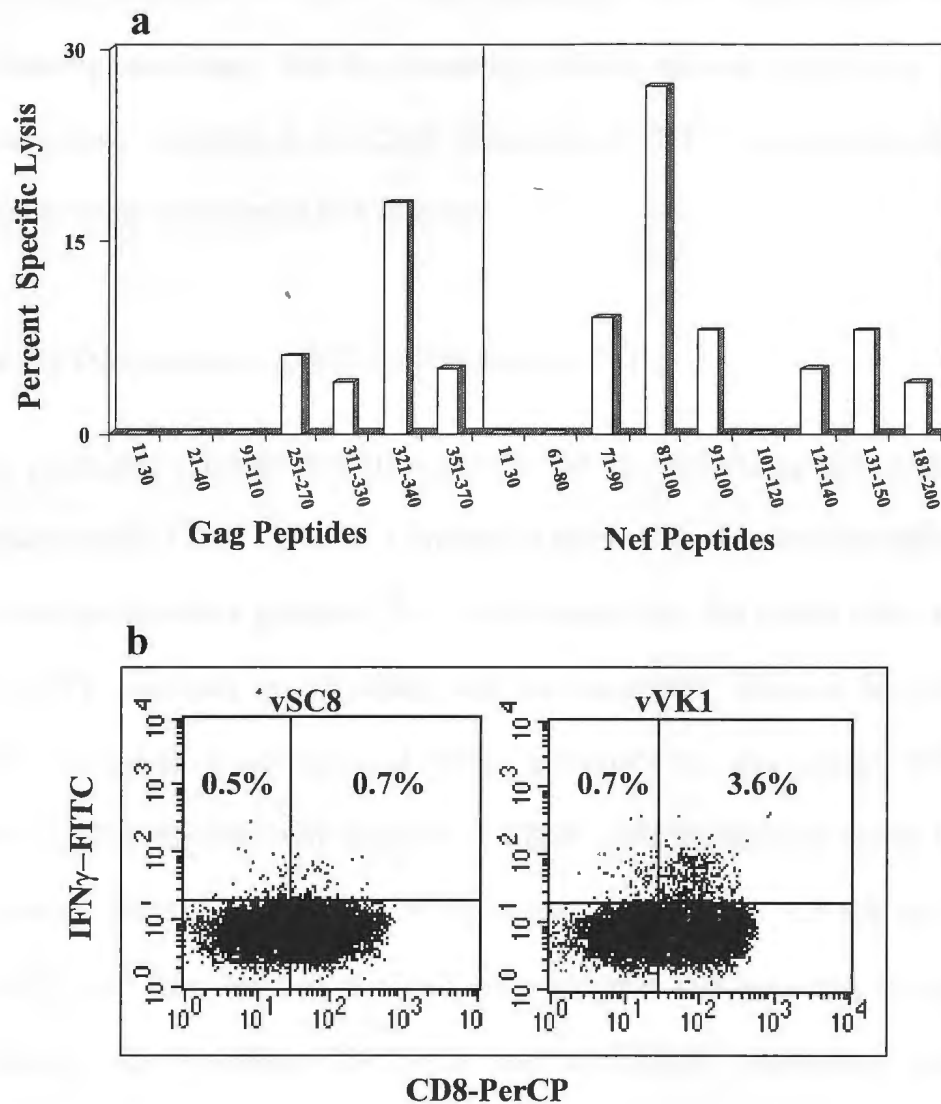


Figure 2. Characterization of emergent HIV-specific CTL. (a) Cytotoxicity of 069 AMLR-generated effector cells against autologous BLCL sensitized with HIV *gag* and *nef* peptides. (b) Intracellular IFN- γ production of 069 AMLR-generated CD8⁺ T cells stimulated by autologous BLCL infected with recombinant vaccinia viruses expressing β -galactosidase (vSC8) or HIV *gag/pol* (vVK1).

proportions of CD8⁺ T cells expressing CD28 at early and late time points for each individual. Subject 028, with the largest increase in CD8⁺ T cell numbers, had a declining proportion of CD8⁺ T cells expressing CD28, while subject 069 showed an increasing proportion. The two remaining subjects showed little change. These results demonstrate variability in the CD28 phenotype of CD8⁺ T cells contributing to immune reconstitution in advanced HIV infection.

3.4.5 CD28 phenotype of HIV-specific memory CTL

We previously reported that HIV-specific CTL present during advanced infection are predominantly CD28 negative, a phenotype associated with terminal differentiation and reduced proliferative potential (7). To determine how this would affect emergent anti-HIV CTL responses in individuals with advanced HIV infection, we investigated the CD28 phenotype of precursor and effector anti-HIV CTL. For subjects 083 (fig. 3a) and 069 (fig. 3b) effectors were depleted of CD28⁺ cells immediately before CTL assays to determine the CD28 phenotype of effector anti-HIV CTL. For subject 028 (fig. 3c), PBMC were first depleted of CD4⁺ cells, and then CD28-positive and negative cells isolated. The resulting CD4⁻CD28⁺ and CD4⁻CD28⁻ populations were stimulated separately and tested for HIV (*gag/pol*)-specific CTL to determine the CD28 phenotype of the precursor anti-HIV CTL proliferating and or differentiating *in vitro* to generate effector anti-HIV CTL. We chose subject 28 for the *in vitro* restimulation of isolated CD28⁺ and CD28⁻ CD8⁺ T cells because of his higher CD8⁺ T cell counts after immune recovery (Table 1) and the need for sufficient starting cell numbers in each subset for

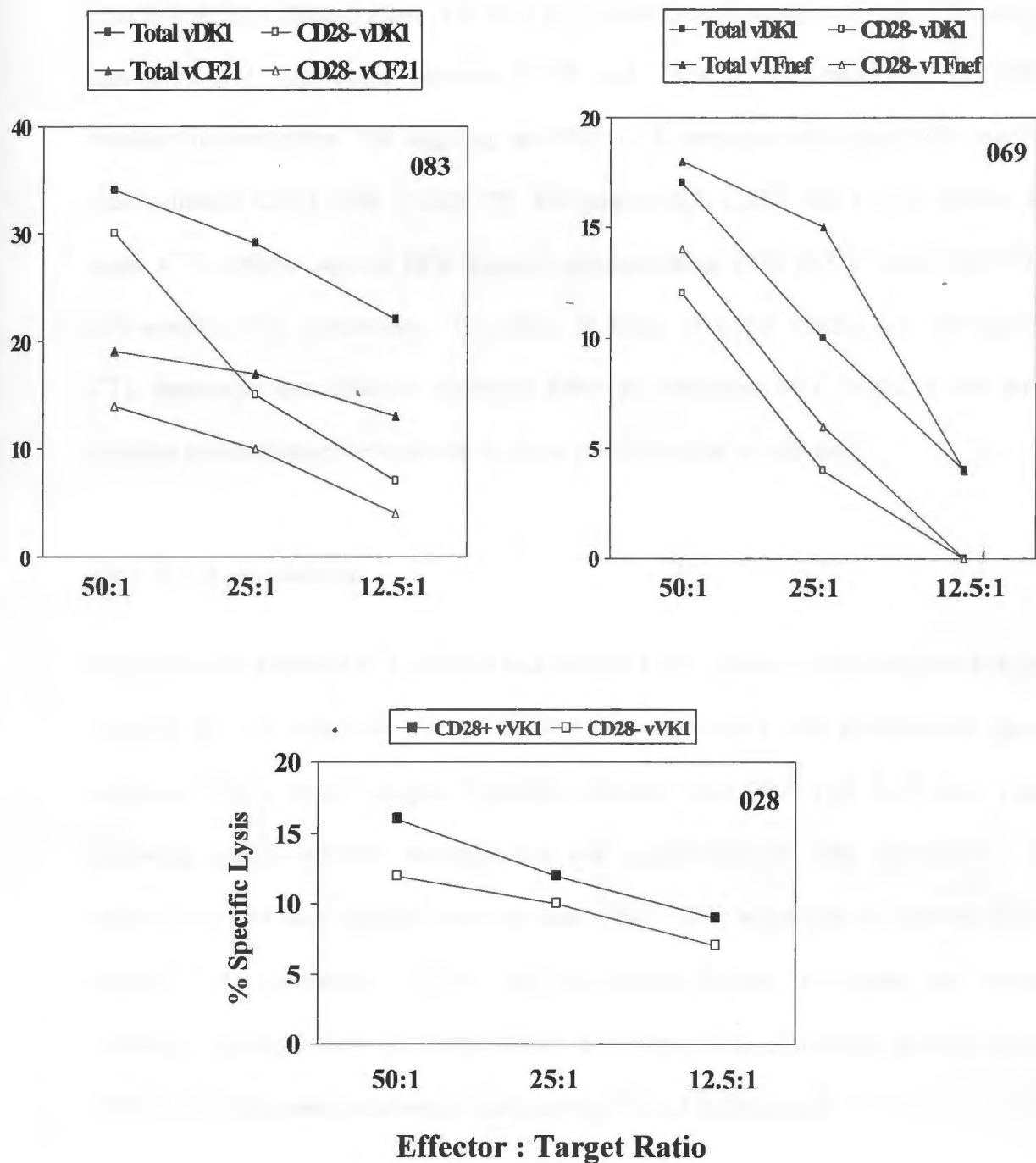


Figure 3. Contribution of CD28⁺ cells to anti-HIV CTL activity. Non-manipulated effector cells (total, dark symbols) and effector cells depleted of CD28⁺ cells (CD28⁻, open symbols) immediately before testing (083, 069) or prior to *in vitro* stimulation (028) were tested for killing of target cells infected with recombinant vaccinia viruses expressing HIV proteins: vDK1, HIV *gag*; vCF21, HIV *pol*; vTFnef, HIV *nef*; vVK1, HIV *gag/pol*. Killing of targets infected with a recombinant vaccinia virus expressing β -galactosidase (vSC8) was subtracted. Subjects are identified in the upper right corner of each graph.

effective *in vitro* restimulation. For all 5 CTL responses examined, effector CTL activity was distributed fairly evenly between CD28⁺ and CD28⁻ T cell subsets (fig. 3). Before immune reconstitution, the ongoing anti-HIV CTL response of subject 083 involved predominantly CD28⁻CD8⁺ T cells (7). For subject 028, CD28⁺ and CD28⁻ cultures had equal CTL activity against HIV *gag/pol*, demonstrating CD8⁺CD28⁺ and CD8⁺CD28⁻ HIV-specific CTL precursors. Therefore, in terms of CD28 phenotype, HIV-specific CTL precursors and effectors emerging following advanced HIV infection and partial immune reconstitution were similar to those present earlier in infection.

3.4.6 T cell proliferation

An association between CTL activity and specific CD4⁺ helper T cell responses has been reported in HIV infection (18). Therefore, we assessed T cell proliferation against mitogen (PHA), recall antigen (*Candida albicans*), and HIV p24 at 2 time points following partial immune reconstitution and suppression of viral replication. No responses to p24 were detected and only one subject (069) responded to *Candida* (fig. 4). However, all responded to PHA with stimulation indices >30 (data not shown). Therefore, although their T cells proliferated in response to a mitogen, antigen specific CD4⁺ T cell responses remained severely compromised in this group.

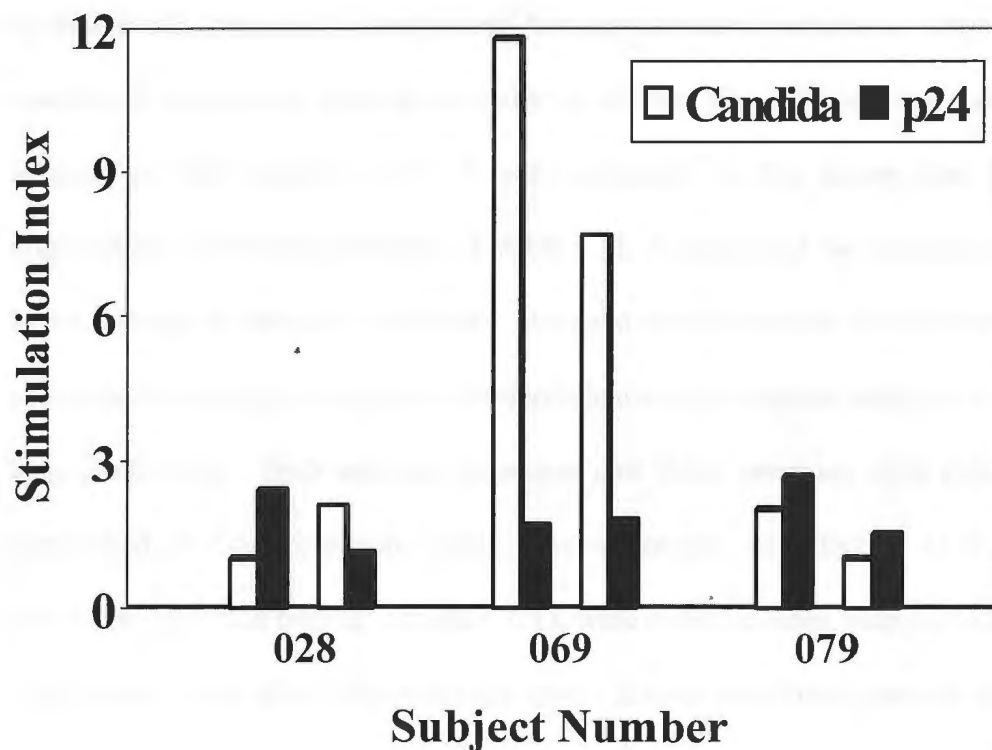


Figure 4. Antigen-specific T cell proliferation following immune reconstitution. At 2 separate time points, specific proliferation in response to *Candida* antigens or HIV p24 was measured. Stimulation indices above 3 were considered to indicate a positive helper T cell response against that antigen.

3.5 DISCUSSION

The ability of HAART to increase T cell numbers and reconstitute anti-HIV CD8⁺ T cell responses at earlier stages of infection is well documented (19-21). However, little is known about the potential to reestablish HIV specific immunity in individuals reaching advanced HIV infection. In this study we examined the impact of effective suppression of viral replication on generalized immune reconstitution and anti-HIV T cell responses in individuals previously progressing through advanced infection. Despite the small number of appropriate subjects available to address this question, we found consistent features of HIV-specific CD8⁺ T cell responses in this group that have general implications for the requirements of stable CTL memory and the prospects for immune-based therapy in advanced infection. The most notable feature of HIV-specific CD8⁺ T cell responses in this group was strict dependence upon ongoing antigenic stimulation for their persistence. Both ongoing responses and those emerging with effective therapy diminished beyond detection within several months of effective viral suppression. Consistent with this finding, anti-HIV CTL were never detected from one subject with an undetectable virus load throughout the study, despite significant immune reconstitution. Our findings clearly indicate that improving the immunological status of individuals previously reaching advanced HIV infection allows emergence of anti-HIV effector CTL. However, at least initially, this response occurs without concurrent generation of stable memory CTL.

Defective generation of memory CTL has also been reported at earlier stages of HIV infection (22), but the absolute short-lived nature of emergent responses following

advanced infection was striking. Since the CD28 phenotype of CD8⁺ CTL has been associated with their stage of differentiation and anticipated lifespan (11,12), we assessed the distribution of CTL and CTL precursors within CD28⁺ and CD28⁻ CD8⁺ T cell subsets. After some degree of immune reconstitution, the distribution of CD28⁺ effectors and precursors was similar to earlier stages of infection, suggesting that cells with the potential for greater proliferation and longer lifespan were being recruited against HIV (7,23). With regard to fine specificity and antigen-specific IFN- γ release, emergent HIV-specific CTL activity was also indistinguishable from earlier stages of infection.

One possible explanation for transient anti-HIV CTL responses in this group is the impact of other immunological factors on activation of CTL and stable generation of memory cells. Differential conditioning of antigen-presenting cells (APC) can determine whether CD8⁺ T cells are activated appropriately or steered towards premature decay (24,25). Circulating dendritic cell precursors are reduced in advanced HIV infection and the effect of HAART on reconstitution of these critical APC is unknown (26,27). In murine lymphocytic choriomeningitis virus (LCMV) infection, CD8⁺ anti-LCMV CTL are activated in the absence of help from CD4⁺ T cells, but without generation of long term CTL memory (28). Furthermore, based on collected data and mathematical modeling, Wodarz et al suggest that with CD4⁺ T cell help, CTL responses persist with very little antigen, yielding long-term control of infection (29). Conversely, without specific CD4⁺ T helper responses, they suggest CTL require high antigen levels to persist and do not control infection in the long term. The transient anti-HIV CTL responses we detected exclusively in concert with breakthroughs in viral replication corroborate previous

findings and this proposed model. Despite substantial increases in total CD4⁺ T cells and strong responses to mitogens, specific CD4⁺ T cell responses to an unrelated recall antigen and to HIV itself were absent or very weak in our subjects. Therefore, the failure to generate long-lived HIV-specific CD8⁺ T memory cells together with effector CTL may reflect HIV-specific CD4⁺ T cell deficiencies. Whether this inability to maintain CD8⁺ T cell memory without replicating virus is confined to individuals reaching advanced HIV infection or common to all chronically-infected subjects, is an important question.

Although there have been suggestions of a narrow window period during which treatment for HIV need be applied to preserve immune responses (30), our data demonstrate that even after HIV infection progresses to extreme T cell lymphopenia, successful treatment and partial immune reconstitution can revive HIV-specific CD8⁺ T cell responses. How the activity we detected *in vitro* reflects *in vivo* activity and what impact the HIV-specific CD8⁺ T cells have *in vivo* at this stage is unknown. However, the consistent association between CTL activity and viral replication and the broadening of CTL activity when virus replication continued suggests the CTL activity detected reflects adaptive responses to *in vivo* viral replication. While revitalization of CD8⁺ T cell responses following HAART in this group extends the potential for immune therapy to individuals with advanced HIV infection, the transience of emergent responses underlines limitations of immune therapy. Allowing limited viral replication through treatment interruption may efficiently activate HIV-specific CD8⁺ T cells, but the risk of eradicating or aborting HIV-specific CD4⁺ T cell responses may outweigh any benefits, especially if the CD8⁺ T cell response is transient. The severity of this risk was recently clarified by

demonstration that HIV selectively infects HIV-specific CD4⁺ T cells (31). If, as both experimental data and theoretical considerations suggest, durable emergent CD8⁺ T cell responses require concurrent help from CD4⁺ T cells, strategies to reactivate CD4⁺ HIV-specific T cell responses should be developed. Remune[™] or other vaccine formulations that activate HIV-specific CD4⁺ T cells without exposure to virus may be useful (32). Likewise, using interleukin-2 (IL-2) to raise CD4⁺ T cell numbers prior to vaccination could increase the possibility of activating HIV-specific helper T cells (33,34). However, there are indications that following the severe CD4⁺ T cell depletion of advanced HIV infection, certain immune defects, such as reduced T cell proliferation and increased T cell activation, persist even after CD4⁺ T cell reconstitution occurs (35,36). The extent of these defects is associated with nadir CD4⁺ T cell counts, indicating that advanced HIV infection imposes a lingering immunological effects not simply overcome by viral suppression and numerical reconstitution of T cell subsets. The nature of these lingering defects and their relationship to nadir CD4⁺ T cell counts warrants further investigation.

In summary, our results show that HAART can restore T cell numbers and the capacity for HIV-specific CTL responses following advanced HIV infection. However, emergent anti-HIV CTL responses were only detected in close proximity to breakthroughs in viral replication. The emergent CTL responses were normal in terms of CD28 phenotype, specificity, cytotoxicity and cytokine release, but rapidly disappeared once viral replication became undetectable. Failure to generate appropriate memory cells under these circumstances may reflect a lack of HIV specific helper T cells. Thus, advanced HIV infection, successfully treated, provides a unique system for identifying important

determinants of memory T cell generation and preservation, and for developing immune therapies beneficial at all stages of HIV infection.

The authors would like to thank the United States National Institute of Allergy and Infectious Diseases (NIAID) AIDS Vaccine Program for providing financial support for this project. The authors would also like to thank the Medical Research Service for providing financial support for this project. The authors would also like to thank the HIV-infected individuals who participated in this study. The study was supported in part by the Canadian Institutes of Health Research (CIHR) grant 14060. AMG is a CIPF scholar and Canada Foundation for Innovation (CFI) scholar. This work was supported by a Canadian Institutes of Health Research (CIHR) grant 14060.

3.6 ACKNOWLEDGEMENTS

The authors would like to thank the United States National Institute of Allergy and Infectious Diseases (NIAID) AIDS reference reagent program for providing recombinant vaccinia viruses and overlapping *gag* peptides, and the Medical Research Council centralised facility for AIDS reagents for providing overlapping *nef* peptides. We also thank the HIV-infected individuals who participated in this study. This study was supported in part by the Canadian Institute for Health Research (CIHR) grant# ROP 37490. MG is a CIHR AIDS scholar and Canada Foundation for Innovation researcher. JG was supported by a Canadian Foundation for AIDS Research (CANFAR)/CIHR doctoral fellowship.

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CHAPTER 4

Lack of CD28 Expression on HIV-specific Cytotoxic T Lymphocytes is Associated With Disease Progression

Running title: CD28 Expression on HIV-specific CTL

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Support: This research was supported by grant # HOP37490 from the Canadian Institutes of Health Research (CIHR). J Gamberg was supported by a Canadian Foundation for AIDS Research (CANFAR)/CIHR PhD fellowship. M Grant held a CIHR AIDS scholar award and is a Canada Foundation for Innovation Researcher.

4.1 Abstract

During HIV infection, CD8⁺ T cells lacking the costimulatory molecule CD28 increase in number and proportion. This accumulation is associated with disease activity and possibly with CD8⁺ T cell dysfunction. In this study, CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells from 41 HIV-infected individuals at various stages of disease were compared in terms of HIV-specific cytotoxicity, TCRβV repertoire diversity, and cytokine production. We found that the CD28 phenotype of anti-HIV CTL evolves in parallel with disease progression and disease activity. Absolute numbers of CD4⁺ T cells and CD4⁺/CD8⁺ T cell ratios progressively decreased in 3 groups with an increasing prevalence of CD28⁻ HIV-specific CTL. Conversely, HIV replication levels progressively increased in parallel with the prevalence of CD28⁻ HIV-specific CTL. Repertoire diversity at the level of TCRβV gene family expression was maintained at normal levels for both CD28⁺ and CD28⁻ T cells at all stages of infection. Diversity at the level of junctional length polymorphism was more restricted in the CD8⁺CD28⁻ T cell population, but this difference remained relatively constant through different stages of infection. Both CD28⁺ and CD28⁻ T cells produced IL-2 and IFN-γ, regardless of disease stage and/or the predominant CD28 phenotype of anti-HIV CTL.

Keywords: CD28, HIV, Cytotoxic T Lymphocytes

4.2 Introduction

The CD28 cell-surface accessory molecule provides secondary signals critical for appropriate activation of naïve CD4⁺ and CD8⁺ T cells¹. At birth, CD28 is expressed on virtually all peripheral blood T cells, suggesting that CD28 expression is the default condition for naïve thymus-derived T cells². In acute viral infections, there is often considerable rapid expansion of CD8⁺CD28⁻ antiviral effector T cells³. Upon antigenic stimulation, these CD8⁺CD28⁻ T cells produce interferon gamma (IFN- γ), but not interleukin-2 (IL-2) and proliferate poorly^{4,5}. This fueled suggestions that CD28⁻ cells can be unresponsive or at least require enhanced costimulation for full activation⁶. The proportion of CD8⁺ T cells expressing CD28 also decreases with age and expanded oligoclonal populations of memory T cells in elderly individuals always lack CD28 expression⁷⁻⁹. This indicates that repeated or prolonged antigenic experience can eventually eliminate CD28 expression even on resting memory cells. These CD8⁺CD28⁻ T cells have shortened telomeres reflecting an extensive history of cell division culminating in replicative senescence¹⁰. Thus, the CD8⁺CD28⁻ T cells in peripheral blood are not a uniform population. While some may be functionally effete remnants of past immunological battles, others may be actively engaged in ongoing cell-mediated anti-viral immune responses. Chronic viral infections, where there is persistent activation of CD8⁺ T cells, are one situation where the proportion of CD8⁺ T cells expressing CD28 could have a variety of meanings and implications in relation to disease activity and progression.

In HIV infection, CD28⁻ T cells often comprise more than 60% of the peripheral blood CD8⁺ T cell population^{5,11}. Expansion of the CD8⁺CD28⁻ T cell subset initially occurs during primary infection and persists throughout the natural course of disease¹². Most CD8⁺CD28⁻ T cells in HIV-infected individuals express activation markers, such as CD38 and HLA-DR, indicative of terminally differentiated effector T cells¹³. However, we and others have shown that there are also CTL precursors within the CD28⁻ population that proliferate and differentiate *in vitro* in response to antigenic stimulation^{14,15}. Identical CD8⁺ CTL clones occur within both the CD28⁺ and CD28⁻ subsets of HIV-infected individuals and radioactive labeling and tracing of T cells showed clearly that CD28⁺ T cells generate CD28⁻ progeny^{16,17}. Effective antiretroviral therapy lowers the proportion of CD8⁺CD28⁻ T cells and shifts the majority of HIV-specific CD8⁺ T cells detectable by tetramer staining from the CD28⁻ to the CD28⁺ population^{18,19}. These data suggest that the predominance of CD8⁺CD28⁻ cells in HIV infection reflects continuous differentiation of CD8⁺CD28⁺ T cells into effector cells in response to chronic viral stimulation. Therefore, the CD8⁺CD28⁻ T cell population present in HIV infection may reflect the mixed accumulation of active effector cells and effete senescent cells. It is important to determine whether characteristics of the expanded CD8⁺CD28⁻ T cell population evolve to reflect the CD8⁺ CTL dysfunction associated with progressive HIV infection. The aim of this study was to compare CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells at different stages of HIV disease progression in terms of their relative contribution to HIV-specific cytotoxicity, TCR β V chain repertoire diversity and cytokine production capacity.

4.3 Materials and Methods

4.3.1 Study subjects

Study participants were recruited through the Infectious Disease Clinic of the St. John's General Hospital, St. John's, NL, Canada. At each visit clinical evaluation was performed, plasma HIV RNA was measured using Amplicor HIV-1 Monitor™ kits (Roche Diagnostic Systems Inc., Mississauga, Ontario, Canada) and peripheral blood lymphocyte (PBL) subsets were assessed by flow cytometry. Ethical approval was obtained from the Memorial University Faculty of Medicine Human Investigation Committee and all participants provided informed consent.

4.3.2 Sample preparation

Peripheral blood mononuclear cells (PBMC) were isolated from acid-citrate dextrose (ACD)-preserved fresh whole blood by density gradient centrifugation with Ficoll-paque lymphocyte separation medium (Pharmacia Chemicals, Dorval, Quebec, Canada). PBMC were washed twice in phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS; Gibco, Grand Island, New York, USA), resuspended in lymphocyte medium (RPMI 1640 with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2×10^{-5} M 2-mercaptoethanol; all from Gibco) and counted. Generation of B lymphoblastoid cell lines (BLCL) from study participants was carried out as previously described¹⁴.

4.3.3 Lymphocyte stimulation

To activate and expand anti-HIV CTL, approximately 1×10^7 PBMC were stimulated by an autologous mixed lymphocyte reaction (AMLR) as previously described¹⁴.

4.3.4 Lymphocyte separations

CD28⁺ lymphocytes were removed from AMLR stimulated effector populations for cytotoxicity assays, and from freshly-isolated PBMC for intracellular flow cytometry staining with purified anti-CD28 (leu28 Beckton Dickinson, Mississauga, Ontario) followed by goat anti-mouse IgG coated magnetic beads (DynaI Inc., Great Neck, New York), as previously described¹⁴. The resulting CD28⁻ (unbound) cells were resuspended at effector to target (E:T) ratios based on starting cell numbers for use in the assays. Flow cytometry revealed that this depletion method removed more than 98% of CD28⁺ T cells (data not shown).

For TCR β V gene repertoire analysis CD4⁺ T cells were depleted from PBMC using magnetic beads coated with anti-human CD4 (DynaI Inc.) at a 10:1 bead-to-target cell ratio prior to CD28⁺ T cell depletion. The resulting CD4⁻CD28⁺ (beaded) and CD4⁻CD28⁻ (unbound) populations were frozen at -70°C for subsequent RNA extraction and analysis. Flow cytometry prior to freezing revealed that depletion resulted in removal of more than 98% of CD4⁺ and CD28⁺ T cells (data not shown).

4.3.5 Cytotoxicity assays

HIV-specific CTL activity was assessed against autologous or human histocompatibility linked antigen (HLA)-matched BLCL targets infected with the following recombinant vaccinia viruses: vVK1 (*gag/pol*); vDK1 (*gag*); vCF21 (*pol*); vPE16 (gp160); vTF*nef* (*nef*) and vSC8 (E. Coli β -galactosidase, control) (all from the NIH AIDS Research and Reference Reagent Program, Rockville, Maryland). Target cells were infected with 15 plaque forming units (pfu)/cell of the desired virus overnight, labeled for 90 minutes with 100 μ Ci Na₂⁵¹CrO₄ (Amersham Life Sciences, Arlington, IL), washed 4 times with PBS containing 1% FBS, counted, and resuspended in lymphocyte medium. Effector and target cells (5000 cells/well) were incubated in duplicate in 96-well culture plates in a final volume of 300 μ l/well at E:T ratios of 50:1, 25:1, and 12.5:1. Minimum and maximum release wells contained targets in medium alone or 1N HCl respectively. After 5 hours incubation, 100 μ l supernatant was removed and counted in a Wallac 1480 gamma counter. Percent killing was determined by the formula:

$$(\text{Test } ^{51}\text{Cr release} - \text{spontaneous release}) \times 100 / \text{maximum } ^{51}\text{Cr release} - \text{spontaneous release}.$$

Spontaneous release was always <25% of maximum release and results were considered positive when % specific ⁵¹Cr release in the test wells was $\geq 10\%$ above control wells.

4.3.6 T cell repertoire analysis

RNA was extracted from CD4⁺CD28⁺ (bead bound) and CD4⁺CD28⁻ (unbound) T cell populations with Trizol (Gibco BRL, Burlington, Ontario) as per the manufacturer's

instructions. TCR β V gene family relative expression levels were assessed by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) as previously described¹⁴. Intrafamily junctional length diversity was assessed by separation of the PCR products on sequencing gels and visualization by silver staining.

4.3.7 Flow Cytometry

To determine the proportion of CD8⁺ T cells expressing CD28, PBMC were washed once in PBS containing 5 mM EDTA and 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri) and incubated for 20 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (Dako Diagnostics, Mississauga, Ontario), phycoerythrin (PE)-conjugated anti-CD28 (Becton Dickinson), and Peridinin Chlorophyll Protein (PerCP)-conjugated anti-CD8 (Becton Dickinson). Samples were washed once after staining, resuspended in 0.5% paraformaldehyde in PBS and analyzed on a FACStarPlus analyzer (Becton Dickinson) after excitation at 488 nm with an argon laser. For intracellular staining, effector cells were incubated overnight at 1×10^6 cells per well in 1 ml medium in 24-well culture plates with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Biomol, Plymouth Meeting, Pennsylvania), 2.5 μ g/ml ionomycin (ICN Biomedicals Inc., Irvine, California), and 10 μ g/ml brefelden A (Sigma Chemical Co.). The next morning each culture well was divided into three aliquots for staining. All were surface stained with PE-conjugated anti-CD8 (Caltag Laboratories, Burlingame, California), fixed with 2% paraformaldehyde in PBS for 15 minutes at 4°C, and permeabilized with 0.2% saponin (Sigma Chemical Co.) in wash buffer for 10 minutes at

4°C. Cells were washed once and stained for 20 minutes at 4°C with FITC-conjugated anti-IFN- γ , FITC-conjugated anti-IL-2, or FITC-conjugated isotype matched controls (all from Caltag Laboratories). Samples were washed again, resuspended in 1% paraformaldehyde in PBS and analyzed on a FACStarPlus analyzer as above.

4.3.8 Statistical Analysis

Normal distribution of data was confirmed by the Kolmogorov-Smirnov test and differences in the mean values of various parameters compared between groups were assessed using Student's *t*-test. Correlation between different continuous variables was assessed by linear regression.

4.4 Results

4.4.1 Subject Characteristics

Forty-one HIV-infected subjects at various stages of disease participated in this study. Most (61%) were receiving antiretroviral therapy at the time of testing. Their CD4⁺ T cell counts ranged from 0-1249 cells/ μ l blood (mean = 398 cells/ μ l) and their CD8⁺ T cell counts ranged from 65-2400 cells/ μ l blood (mean = 903 cells/ μ l). Fourteen individuals (34%) had <200 CD4⁺ T cells/ μ l blood, 18 (44%) had between 200 and 500 CD4⁺ T cells/ μ l blood, and 9 (22%) had more than 500 CD4⁺ T cells/ μ l blood. Virus load measurements ranged from undetectable (<1.7 log₁₀ copies HIV RNA/ml plasma) to 5.9 log₁₀ copies/ml plasma (mean = 3.7 log₁₀ copies/ml). The proportion of CD8⁺ T cells lacking CD28 expression ranged from 17%-82% (mean = 55%). CD28 negative cells accounted for >60% of the CD8⁺ T cell population in 21 subjects. Therefore, our study cohort represented a broad cross-section of HIV disease status in terms of both ongoing disease activity and the cumulative impact of disease progression on T cell subsets²⁰.

4.4.2 Cytotoxicity Assays

The CD28 phenotype of anti-HIV CTL generated by *in vitro* stimulation was examined for 30 subjects. Figure 1 shows HIV-specific CTL assay results for representative subjects from each of 3 groups distinguished by the predominant CD28 phenotype of their anti-HIV CTL. For 11 of the 30 individuals, HIV-specific CTL were predominantly CD28⁺ (table 1, group 1) as HIV-specific cytotoxicity >10% above background was detected within the unmanipulated cell population only (figure 1a). Twelve individuals

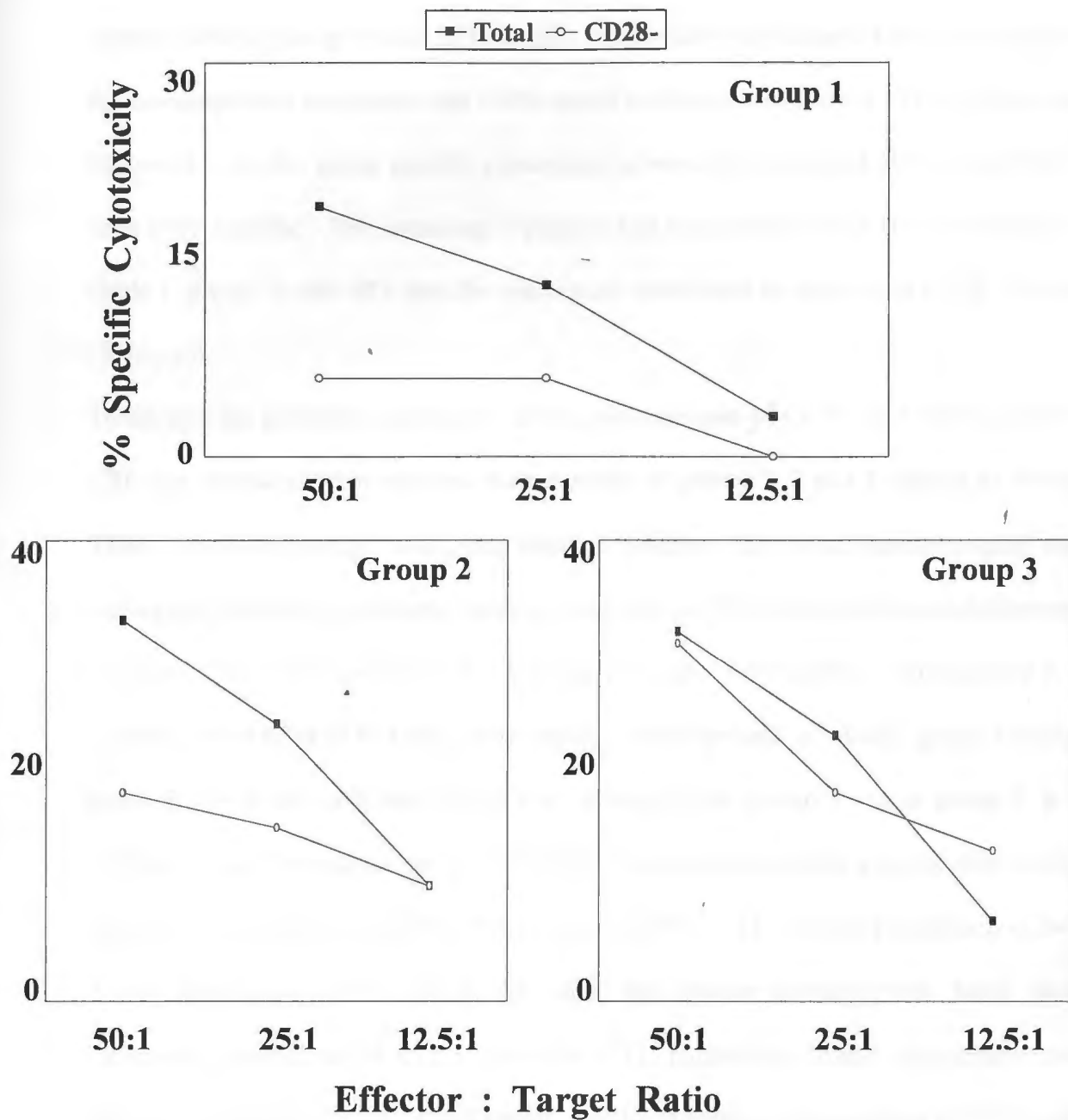


Figure 1. Anti-*gag/pol* activity of unmanipulated (solid symbols) and CD28⁺ T cell-depleted (open symbols) populations for 1 representative individual from each of group 1 (predominantly CD28⁺ CTL), group 2 (CD28⁺ and CD28⁻ CTL), and group 3 (predominantly CD28⁻ CTL). Background lysis (cytotoxicity against targets infected with VsC8) has been subtracted.

had anti-HIV CTL distributed equally between the $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets (table 1, group 2) with HIV-specific cytotoxicity >20% above background within the unmanipulated population and >10% above background within the $CD28^-$ population (figure 1b). In this group specific cytotoxicity always fell by at least 10% when $CD28^+$ cells were depleted. The remaining 7 subjects had exclusively $CD28^-$ HIV-specific CTL (table 1, group 3) with HIV specific cytotoxicity unaffected by removal of $CD28^+$ T cells (figure 1c).

To address the potential significance of the predominance of $CD28^-$ or $CD28^+$ anti-HIV CTL, we compared other relevant characteristics of groups 1, 2 and 3 defined as above. Table 1 shows the range, mean, and standard deviation for several immunological and virological parameters compared between the 3 groups. We found significant differences in mean $CD4^+$ T cell counts (group 1 versus group 2, $p = 0.007$, group 1 versus group 3, $p = 0.003$), mean $CD4/CD8$ T cell ratios (group 1 versus group 2, $p = 0.002$, group 1 versus group 3, $p = 0.003$) and mean virus load measurements (group 1 versus group 3, $p = 0.00008$, group 2 versus group 3, $p = 0.0008$). These results outline a strong relationship between the predominant $CD28$ phenotype of anti-HIV CTL, disease progression ($CD4^+$ T cell count and $CD4^+/CD8^+$ T cell ratio) and disease activity (virus load), with increasing proportions of $CD28^-$ anti-HIV CTL paralleling disease progression and activity. Neither total $CD8^+$ T cell counts, nor the proportion of circulating $CD8^+$ T cells expressing $CD28$ differed significantly between any of the 3 groups. Each of the parameters compared between groups 1, 2 and 3 was tested for correlation with the percentage of $CD28^-$ anti-HIV CTL by linear regression and significance determined from the correlation coefficient (r). For the entire group of 30 individuals, we found

Table 1. Characteristics of groups of individuals with differing proportions of CD28⁺ HIV-specific CTL

	Group 1* n = 11		Group 2 n = 12		Group 3 n = 7	
Parameter	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD
CD4 ⁺ T cells/μl blood	149-1249	620 ± 342	14-646	320 ± 182	0-474	181 ± 179
CD8 ⁺ T cells/μl blood	227-1240	774 ± 315	65-1932	1012 ± 622	215-1304	680 ± 381
CD4:CD8 ratio	0.3-1.9	0.9 ± 0.5	0.2-1.0	0.4 ± 0.3	0-0.7	0.3 ± 0.3
Virus load [†]	1.7-3.8	3.0 ± 0.5	1.7-4.7	3.3 ± 0.6	3.6-5.9	4.6 ± 0.9
%CD8 ⁺ T cells CD28 ⁺	37-77	49 ± 14	18-74	43 ± 15	25-61	38 ± 14
% HIV-specific lysis [‡]						
Total	12-27	19 ± 4.9	17-54	32 ± 13	12-45	26 ± 12
CD28-negative	0.0-10	4.8 ± 3.5	10-41	22 ± 11	14-50	26 ± 13

*Group 1 is composed of subjects with HIV-specific CTL that are predominantly CD28⁺, group 2 of subjects with both CD28⁺ and CD28⁻ CTL and group 3 of subjects with predominantly CD28⁻ CTL.

[†]Virus load is expressed as log₁₀ HIV RNA copies/ml plasma.

[‡]The mean ± SD and range of % HIV-specific lysis for each group at an E:T ratio of 50:1 is shown for both unmanipulated (total) and CD28⁺ T cell-depleted (CD28-negative) effector cell populations.

significant correlations between the percentage of CD28⁻ anti-HIV CTL and CD4⁺ T cell counts ($r = -0.52$, $p < .01$), CD4:CD8 ratio ($r = -0.55$, $p < .01$) and HIV plasma virus load ($r = 0.64$, $p < .001$). Figure 2a-c graphically shows the relationship between the relevant parameters and percentage of CD28⁻ anti-HIV CTL with the line of best fit superimposed over scatter plots. The lack of a statistically significant relationship between the proportion of circulating CD8⁺ T cells that were CD28⁺ and the predominant CD28 phenotype of restimulated anti-HIV CTL suggests that accumulation of CD8⁺CD28⁻ T cells in the peripheral blood of HIV-infected individuals does not simply reflect activation of anti-HIV CTL.

4.4.3 TCR β chain V gene family expression levels

To determine whether any differences in TCR β chain V gene family usage by CD28⁺ and CD28⁻ T cells were associated with the different cytotoxicity patterns, we compared CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells by RT-PCR. For most of the 14 individuals whose CD8⁺ T cells were analyzed, a pattern similar to figure 3a was evident, in that 1 or 2 families within the CD8⁺CD28⁻ population were expanded compared to their CD8⁺CD28⁺ counterparts. The affected families differed between individuals such that overall there was very little difference between CD28⁺ and CD28⁻ expression levels of each TCR β V family (figure 3b). Only relative TCR β V9 levels differed significantly when the mean TCR β V family levels for all 14 individuals were compared (11.7% for CD28⁺, 18.1% for CD28⁻, $p < 0.01$).

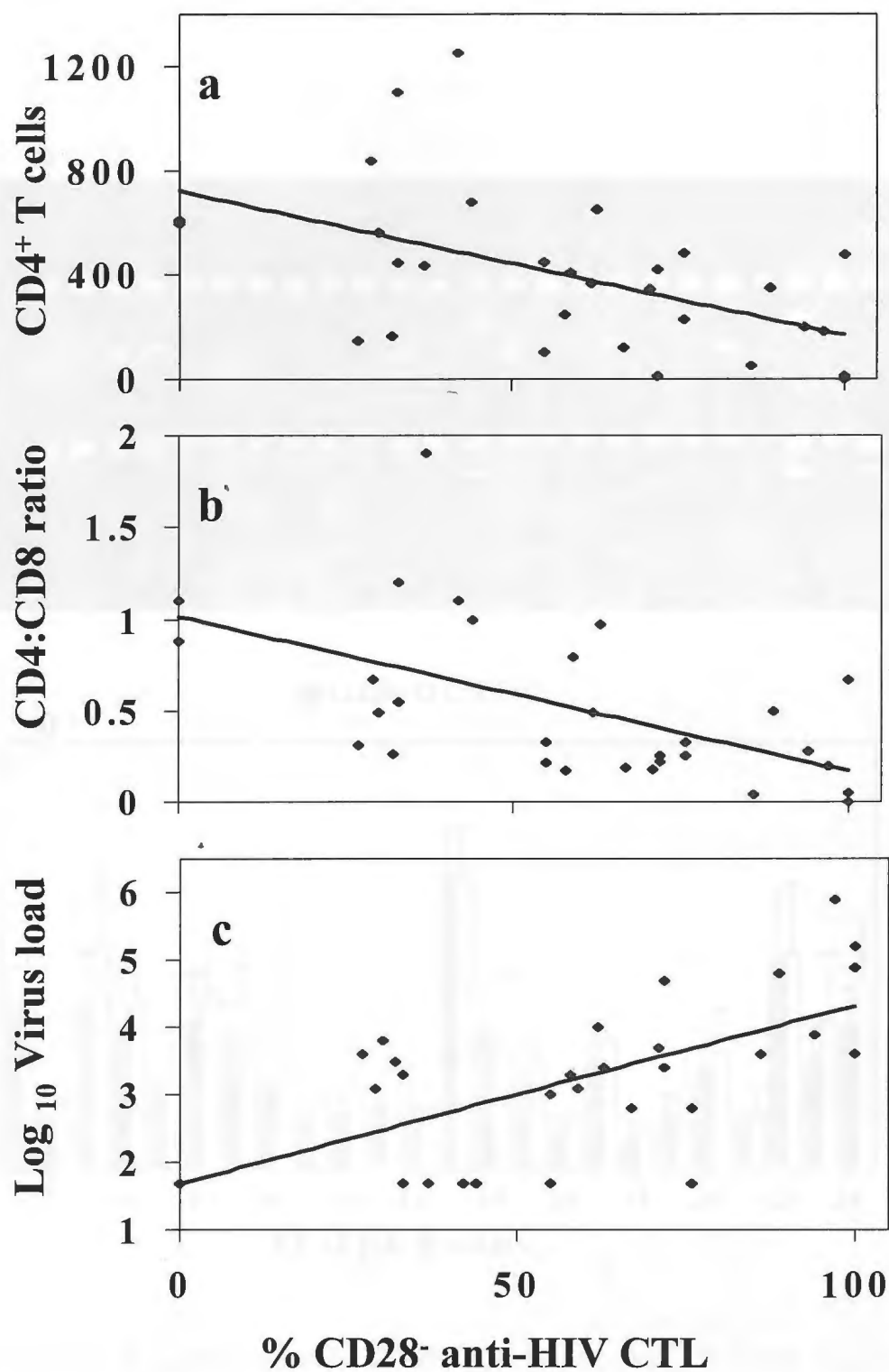


Figure 2. Correlation between percentage CD28 negative anti-HIV CTL and markers of HIV disease activity and progression. Scatter plots of percentage CD28 negative anti-HIV CTL versus CD4⁺ T cell counts/ μ l peripheral blood (a), CD4/CD8 T cell ratios (b), and Log₁₀ plasma HIV load (c) are shown together with the line of best fit in each case.

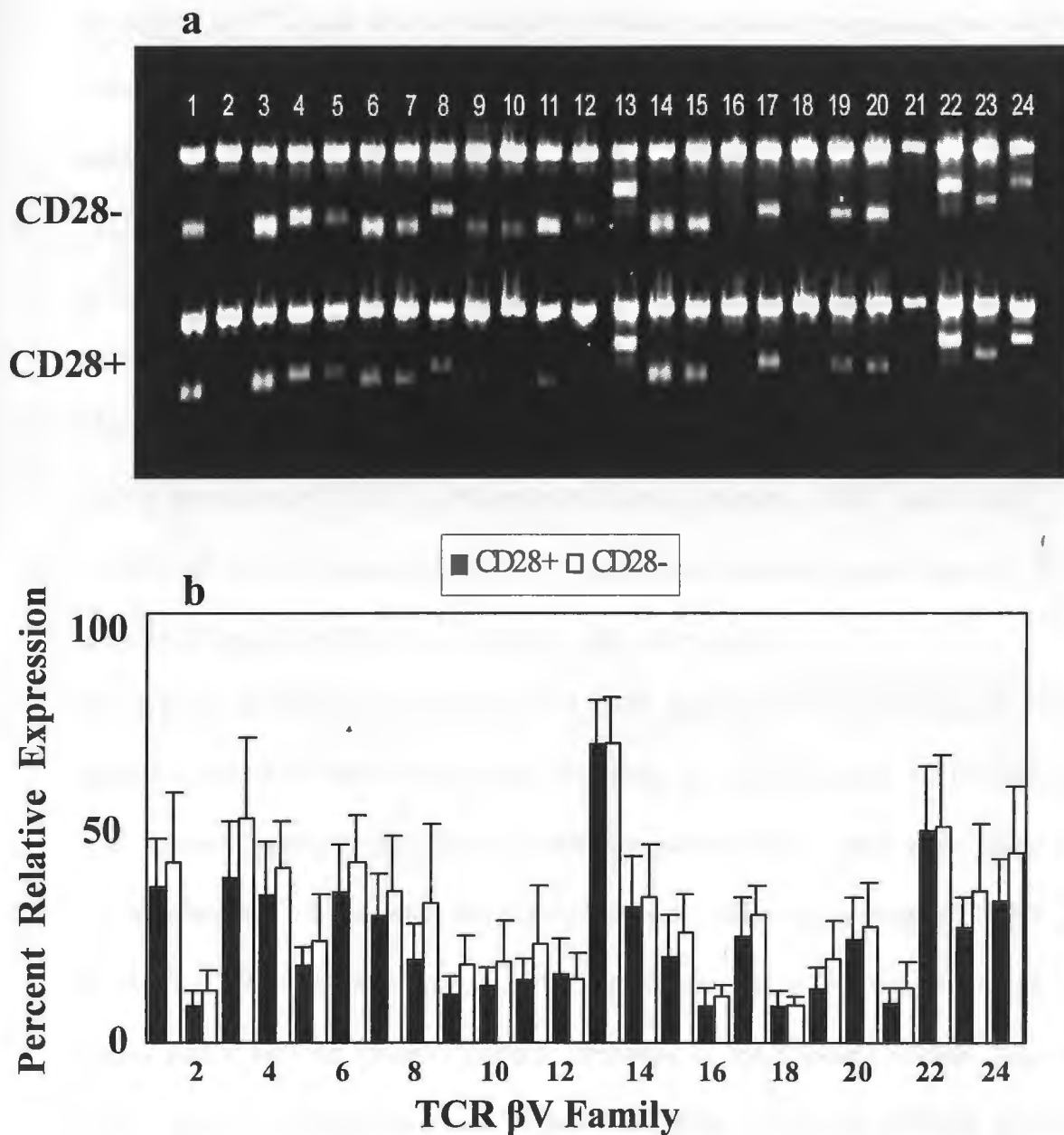


Figure 3. (a) RT PCR analysis of the 24 TCR β V gene families for CD8⁺CD28⁻ (top) and CD8⁺CD28⁺ (bottom) T cells from 1 representative HIV-infected subject. (b) Mean relative TCR β V family expression levels (\pm standard deviation) for CD8⁺CD28⁺ (dark bars) and CD8⁺CD28⁻ (light bars) T cells from 14 HIV-infected subjects. The intensity of each β V band was expressed as a percentage of the α C control band intensity for standardization and inter-individual comparison of relative TCR β V intensity levels.

Since HIV-specific CTL analysis revealed strong associations between the CD28 phenotype of CTL and several parameters related to disease progression, we wanted to determine whether individuals with low CD4⁺ T cell counts and high levels of virus replication showed greater variability in TCR β V family expression levels between CD28⁺ and CD28⁻ T cells compared to subjects with higher CD4⁺ T cell counts and undetectable levels of virus replication. Five of the 14 individuals analyzed had <200 CD4⁺ T cells/ μ l blood and >4.5 log₁₀ copies HIV RNA/ml blood, while another 5 had >300 CD4⁺ T cells/ μ l blood and undetectable levels of virus replication. Within the former group, only TCR β V6 differed significantly between CD28⁺ and CD28⁻ T cells (27.4% and 40.9% respectively, $p < 0.01$), while the levels of none of the 24 TCR β V families differed significantly for subjects in the latter group.

For a more detailed examination of the TCR repertoire, junctional length diversity analysis within 7 TCR β V families was performed. In all cases tested, the CD8⁺CD28⁺ T cells showed the typical intrafamily distribution pattern of 5-7 bands with central bands the most intense²¹. In contrast, length polymorphism within the expressed TCR β V genes of the CD8⁺CD28⁻ T cells was lesser in many instances, with fewer individual bands visible and a skewed intensity pattern compared to the standard normal distribution (figure 4). Of 10 subjects tested, 8 had perceptible intrafamily TCR β V differences between CD28⁺ and CD28⁻ T cells for at least one family. However, the extent of the observed differences did not appear to be associated with either disease stage or the CD28 phenotype of anti-HIV CTL.

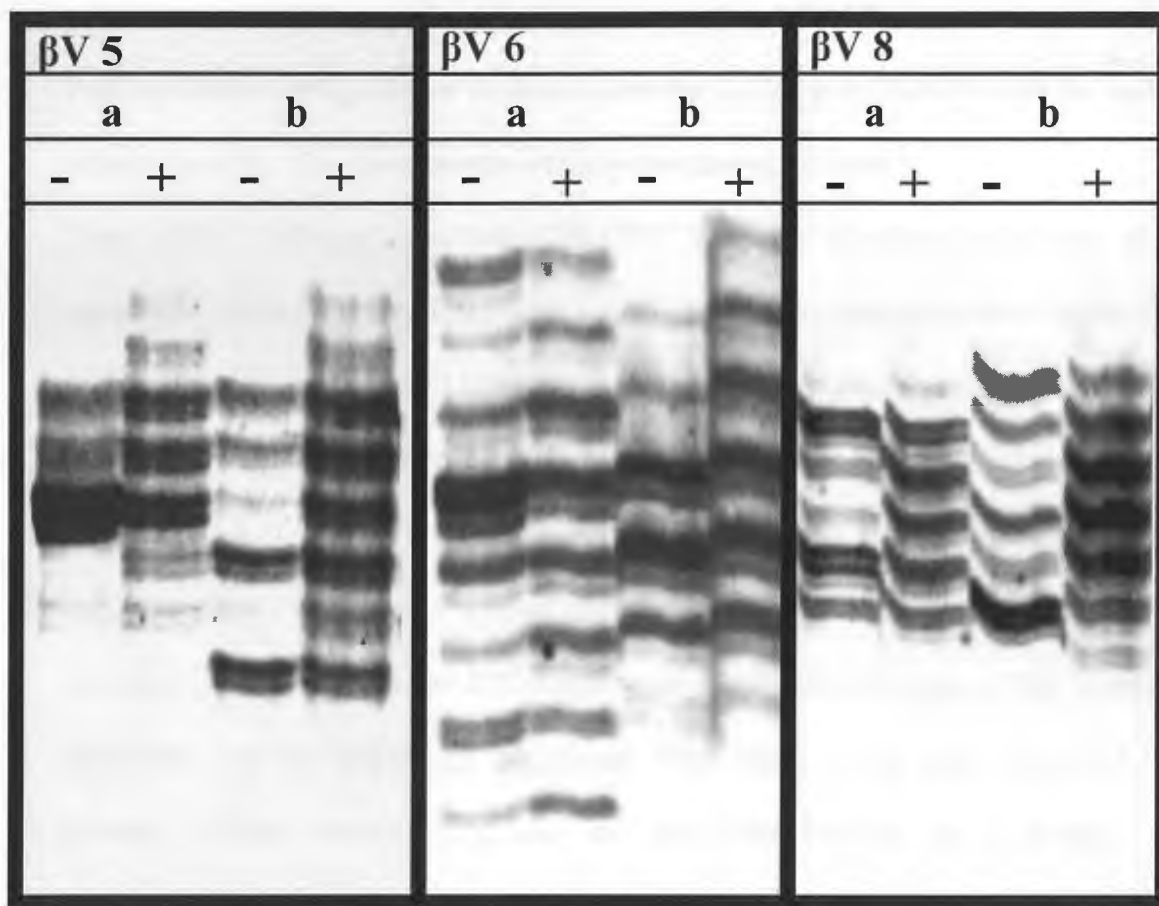


Figure 4. TCR βV gene intrafamily junctional length diversity analysis of CD8⁺CD28⁺ (+) and CD8⁺CD28⁻ (-) T cells. Products from 3 TCR βV families ($\beta V 5$, $\beta V 6$, $\beta V 8$) of 2 representative HIV-infected subjects (a and b) separated on sequencing gels and visualized by silver staining are shown.

4.4.4 Intracellular cytokine analysis

To investigate potential functional differences between $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells related to cytokine production, both unmanipulated and $CD28^-$ T cell populations were tested for IL-2 and IFN- γ production in response to PMA/ionomycin stimulation. Both cytokines were produced to some extent by $CD28^+$ and $CD28^-$ T cells for each of 9 subjects studied. One representative example is shown in figure 5.

Three of the 9 subjects tested had <200 $CD4^+$ T cells/ μ l blood and high levels of virus replication, while virus replication was suppressed below detectable levels and $CD4^+$ T cell counts were >400 cells/ μ l blood for the other 6 individuals. Table 2 shows the range, mean, and standard deviation of the percentage of PMA/ionomycin stimulated $CD8^+$ T cells secreting IL-2 and IFN- γ for both total PBMC and $CD28^-$ PBMC populations in these 2 groups. Although it seemed apparent that fewer $CD28^-$ cells from individuals with low CD4 counts secreted IFN- γ than those of subjects with higher CD4 counts, this difference was not statistically significant when mean values were compared. The number of cells secreting IL-2 also did not differ between the 2 groups. Both $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells produced IFN- γ and IL-2 in response to PMA/ionomycin stimulation.

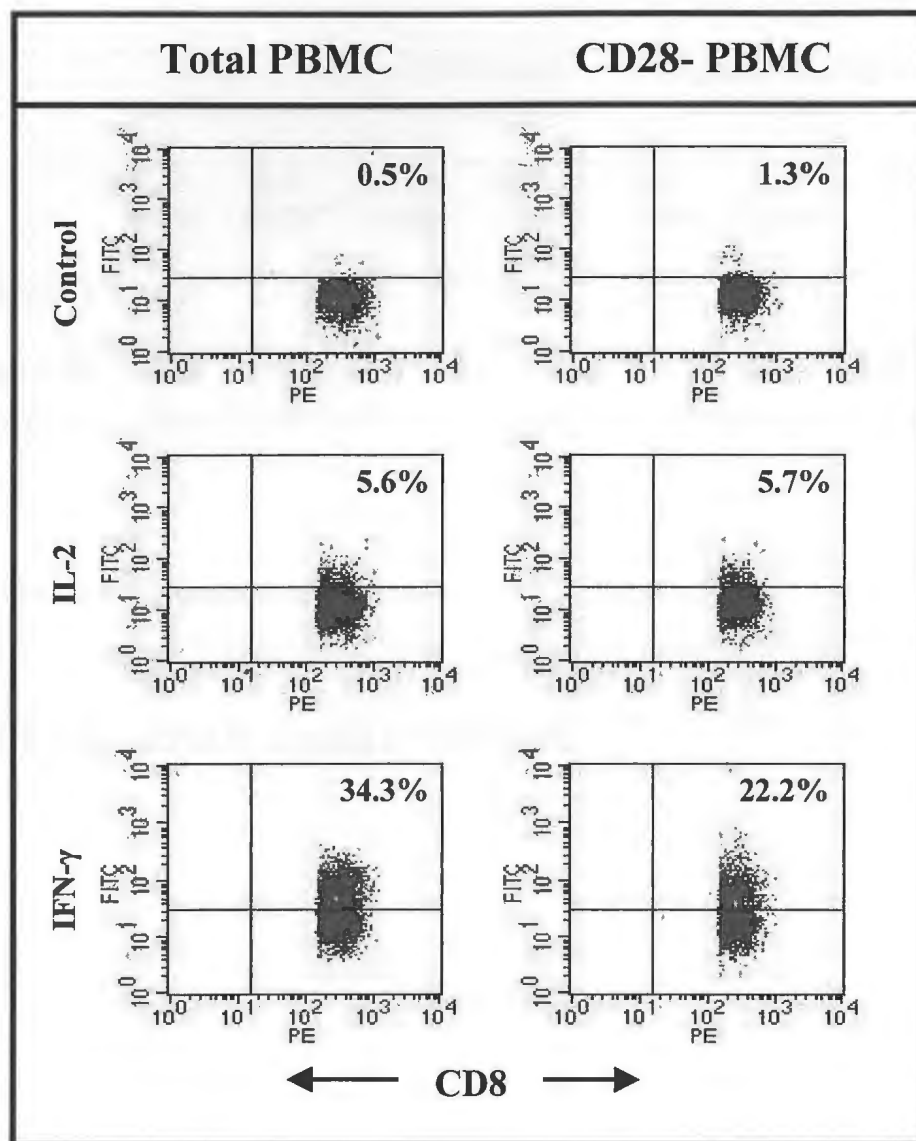


Figure 5. Intracellular flow cytometry results for IL-2 (middle panels) and IFN- γ (lower panels) production by unmanipulated (left) and CD28-depleted (right) PMA-stimulated PBMC. The upper panels show isotype matched negative controls. We gated on CD8⁺ cells for analysis and only CD8 high cells are shown in the flow cytometry plots to exclude CD8⁺CD28⁻ natural killer cells.

Table 2. IL-2 and IFN- γ production from T cells of individuals with and without characteristics of progressive HIV infection

	3 subjects with low CD4 counts and high levels of virus replication				6 subjects with high CD4 counts and undetectable virus replication			
	IL-2 Total*	IL-2 CD28 [†]	IFN- γ Total	IFN- γ CD28 [†]	IL-2 Total	IL-2 CD28 [†]	IFN- γ Total	IFN- γ CD28 [†]
Range (%)	2 – 4	2 – 5	4 – 19	4 – 11	2 – 15	2 – 11	7 – 50	4 – 49
Mean \pm SD	3 \pm 1	4 \pm 1.5	11 \pm 8	7 \pm 4	6 \pm 5	4 \pm 3	25 \pm 17	21 \pm 16

*Total refers to unmanipulated PBMC.

[†]CD28[†] refers to PBMC depleted of CD28⁺ cells.

4.5 Discussion

Both the number and proportion of $CD8^+$ T cells lacking surface CD28 expression increase dramatically during HIV infection³⁻⁶. To explore the possibility of a relationship between expansion of this subset and the $CD8^+$ T cell dysfunction that parallels disease progression, we determined the predominant CD28 phenotype of HIV-specific CTL from individuals at various stages of chronic infection. Three patterns were readily distinguishable. Anti-HIV CTL were either predominantly $CD28^+$, more or less equally distributed between $CD28^+$ and $CD28^-$ subsets, or predominantly $CD28^-$. There were substantial differences in various immune parameters compared between these 3 groups of HIV-infected subjects distinguished on the basis of the predominant CD28 phenotype of their anti-HIV CTL. Mean $CD4^+$ T cell numbers and mean $CD4^+/CD8^+$ T cell ratios progressively decreased as the predominance of $CD28^-$ anti-HIV CTL increased in groups 1 through 3. Conversely, HIV replication levels progressively increased in groups 1 through 3 in parallel with increasing predominance of $CD28^-$ anti-HIV CTL. We also observed significant inverse correlations between the percentage of anti-HIV CTL that were $CD28^-$, $CD4^+$ T cell counts and $CD4^+/CD8^+$ T cell ratios. There was a highly significant direct correlation between the percentage of anti-HIV CTL that were $CD28^-$ and HIV plasma virus load levels. Since $CD4^+$ T cell numbers, $CD4^+/CD8^+$ T cell ratios, and virus load are related to HIV disease stage and/or activity, this data indicates that the CD28 phenotype of anti-HIV CTL also reflects HIV disease stage and activity. Thus, as has been previously reported for the circulating $CD8^+$ T cell population as a whole, the predominance of $CD28^-$ anti-HIV CTL parallels disease progression²².

To investigate the possible basis for the observed association between HIV disease stage and CD28 phenotype of anti-HIV CTL, we compared CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells at different stages of HIV disease in terms of their TCRβV repertoire diversity and cytokine production profile. We speculated that if disease progression directly related to the accumulation of CD28⁻ CTL, decreased diversity in the TCRβV repertoire and/or defective cytokine production within the CD28⁻CD8⁺ T cell subset of individuals with more advanced disease could be involved. For examination of TCRβV diversity, both inter and intrafamily diversity was studied. At the family level, most individuals demonstrated selective CD28⁻ T cell expansions within one or 2 families, regardless of disease stage. Since the affected families differed between individuals, no overall difference was detected between CD28⁺ and CD28⁻ TCRβV family expression levels. However, since the methodology utilized was semi-quantitative and addressed the CD8⁺ T cell repertoire as a whole rather than the HIV-specific repertoire, it is possible that a fully quantitative more targeted approach might detect differences between CD28⁺ and CD28⁻ TCRβV family expression levels for individuals at different stages of disease progression.

When intrafamily junctional length diversity was assessed, the CD8⁺CD28⁻ cells often showed less diversity and/or a skewed distribution pattern compared to their CD8⁺CD28⁺ counterparts. However, there was again no notable difference in the extent of CD28⁻ T cell junctional length diversity observed between individuals at early or more advanced stages of disease. These data indicate that, at family and intrafamily levels, TCRβV repertoire diversity is maintained at roughly normal levels for both CD28⁺ and CD28⁻ T

cells, regardless of disease stage and/or CD28 expression of HIV-specific CTL. It should be noted that TCR β V diversity analysis was also assessed on the total CD8⁺ T cell population and these results don't necessarily reflect what is occurring in the HIV-specific CTL population. In any case, our results are consistent with the CD8⁺CD28⁻ T cells representing that fraction of the entire CD8⁺ T cell repertoire either currently or previously activated and recruited into the effector cell population. Previous studies showed that the dense oligoclonal bands within the CD28⁻ population were comprised of a dominant clone also present in the CD28⁺ population¹⁶.

We also examined IFN- γ and IL-2 production by CD28⁺ and CD28⁻ T cells stimulated with PMA/ionomycin and found that both subsets secreted these cytokines, regardless of disease stage. The percentage of CD28⁻ T cells from individuals with advanced disease producing IFN- γ after stimulation appeared to be considerably less than from individuals at earlier stages of disease, however, this difference was not statistically significant. Production of IL-2 by CD8⁺CD28⁻ T cells was low for all individuals studied with no differences observed on the basis of disease stage.

The results of this study demonstrate an association between accumulation of CD28⁻ HIV-specific CTL and disease progression. Mechanistically, this association cannot be readily attributed to disease related degeneration of CD8⁺CD28⁻ T cells in terms of either their TCR β V family repertoire diversity or ability to produce relevant cytokines such as IFN- γ or IL-2. The expanded CD28⁻ T cell population in HIV infection consists at least partly of activated, effector anti-HIV CTL and identical expanded clones occur within both CD28⁺ and CD28⁻ subsets^{13,16}. This suggests that for the most part, CD8⁺CD28⁺ and

CD8⁺CD28⁻ T cells in peripheral blood are phenotypic variants of the same population, with the CD28⁻ effector T cells being recruited from CD28⁺ precursors and accumulating due to unresolved antigenic stimulation¹⁷. Several investigators reported that CD28⁻ T cells are anergic, which can be reversible, while others reported that loss of CD28 expression reflects telomere shortening and is one of the main genetic alterations associated with irreversible growth arrest^{6,8,10}. Therefore, at any particular time point in HIV infection, the CD8⁺CD28⁻ T cell population should be comprised of effector cells engaged in the ongoing immune response and senescent or “spent” cells left over from past responses. As disease progresses, we would expect the mixture to represent more spent cells and fewer effector cells. The increasing predominance of CD28⁻ anti-HIV CTL that we observed in parallel with disease progression suggests that recruitment from the CD28⁺ population has limits, but the character of the CD8⁺CD28⁻ T cells as reflected by TCRβV repertoire diversity or ability to produce IFN-γ and IL-2 didn’t change significantly with disease progression. The lack of apparent changes in repertoire diversity may reflect rapid turnover of the CD28⁻ population driven by HIV replication at all stages of chronic HIV infection. In this case, although individual clones within the CD28⁻ population would continuously evolve, overall diversity could remain fairly constant. The ability of CD8⁺CD28⁻ T cells to produce IFN-γ is well recognized for activated effector cells, but a decreased capacity for cytokine production with advanced disease might be expected if this were associated with a shift to a more predominantly spent CD8 population²³. No significant decrease in IFN-γ production by CD8⁺CD28⁻ T cells from individuals with advanced disease was observed. However, we used a highly

potent stimulus bypassing the role of surface molecule engagement, rather than antigen-specific stimuli, which could also explain the unexpected IL-2 production by CD8⁺CD28⁻ T cells⁴. However, even if nominal antigenic stimulation is an insufficient trigger, this demonstrates that CD8⁺CD28⁻ T cells can produce IL-2.

In summary, we found a clear relationship between HIV disease progression as measured by CD4⁺ T cell counts, HIV disease activity as measured by virus load, and the proportion of HIV-specific CTL expressing CD28. As HIV disease progresses and viral replication increases, the HIV-specific CTL response appears to become stressed and eventually spent in terms of its ability to recruit new effector cells from the CD8⁺CD28⁺ T cell population. Our data also indicate that the relationship between HIV disease progression and accumulation of CD8⁺CD28⁻ T cells reflects the stress imposed on the immune system by HIV rather than a characteristically dysfunctional CD8⁺ T cell response. This suggests that effective immune responses retain a reserve of potential effector cells within the CD8⁺CD28⁺ T cell population, whereas ineffective responses inexorably shift to CD8⁺CD28⁻ predominance.

4.6 Acknowledgements

The authors would like to thank the United States National Institute of Allergy and Infectious Diseases (NIAID) AIDS reference reagent program for providing recombinant vaccinia viruses. We are also grateful to the HIV-infected individuals who participated in this study.

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CHAPTER 5

Factors Related to Levels of HIV-specific Cytotoxic T Lymphocyte Response

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Abstract: The purpose of this study was to determine the factors related to the levels of HIV-specific Cytotoxic T Lymphocyte (CTL) response in HIV-infected individuals. The study was conducted in a cohort of HIV-infected individuals in the Province of Quebec, Canada.

Results: The study included 100 HIV-infected individuals. The levels of HIV-specific CTL response were measured using a standard assay. The results showed that the levels of HIV-specific CTL response were significantly higher in individuals with a higher CD4⁺ count and a lower viral load. The levels of HIV-specific CTL response were also significantly higher in individuals with a higher duration of infection and a higher level of adherence to antiretroviral therapy.

Conclusion: The results of this study suggest that the levels of HIV-specific CTL response are related to the CD4⁺ count, viral load, duration of infection, and adherence to antiretroviral therapy.

Keywords: HIV, CTL, CD4⁺ count, viral load, duration of infection, adherence to antiretroviral therapy.

CHAPTER 5

Factors Related to Loss of HIV-specific Cytotoxic T Lymphocyte Activity

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Support: This research was supported by grant# HOP37490 from the Canadian Institutes of Health Research (CIHR). J. Gamberg was supported by a Canadian Foundation for AIDS Research/CIHR PhD fellowship. L. Barrett holds a CIHR MD/PhD fellowship. M. Grant held a CIHR AIDS scholar award and is a Canada Foundation for Innovation Researcher.

Running title: Loss of HIV-specific CTL

Text = 3258 words

5.1 Abstract

Introduction: To identify factors associated with loss of *in vitro* stimulated anti-HIV CTL activity, we compared immunological, virological and other characteristics of one group of individuals who sustained anti-HIV CTL activity for prolonged periods with viral replication suppressed below detectable levels to another group that lost anti-HIV CTL activity under the same circumstances.

Methods: Forty-four individuals, all but 1 receiving HAART or combination therapy, were followed up to 56 months. Virus load, lymphocyte counts, CD28 expression on CD8⁺ T cells, *in vitro* restimulated HIV-specific CTL and T cell proliferation were assessed at regular intervals.

Results: Twenty individuals with consistently detectable HIV replication and 17/24 individuals with sustained suppression of HIV replication maintained anti-HIV CTL responses throughout. As a group, the 7 who lost anti-HIV CTL were older, had weaker baseline anti-HIV CTL activity, higher historical virus loads, lower historical and contemporary CD4⁺ T cell counts and a lower percentage of CD8⁺ T cells expressing CD28. Multivariate analysis suggested CD4⁺ T cell counts and anti-HIV CTL amplitude at study onset were independently associated with CTL loss in these 7 individuals, as was percentage of CD8⁺ T cells expressing CD28 at study's end. There was a significant direct correlation between nadir CD4⁺ T cell counts and duration of anti-HIV CTL persistence after suppression of viral replication.

Conclusions: Most HIV-infected individuals retain CD8⁺ anti-HIV CTL with *in vitro* proliferative potential, even when antigen is limited. Those who lose HIV-specific CTL

responses generally show past or current evidence of severe disease progression or activity.

Keywords: Immune memory, Cytotoxic T lymphocytes, Virus load, HAART

5.2 Introduction

Anti-HIV CTL are critical for immune suppression of HIV replication, however, the initially vigorous CD8⁺ T cell response against HIV incompletely controls replication. Without effective intervention, the breadth of anti-HIV CTL activity decreases gradually with progressive disease [1-5]. Highly active antiretroviral therapy (HAART) is central to effective intervention, but cumulative toxicity and multi-drug resistance can limit the long-term success of pharmacological intervention. Therefore, improving immune control of HIV replication remains an important therapeutic objective. While even temporary pharmacological suppression should augment control of HIV replication by pre-existing immunity, virus loads rapidly rebound to pretreatment levels following most treatment interruptions [6]. Previous studies showed that, after HAART, the frequency of circulating, activated HIV-specific CD8⁺ T cells falls rapidly in parallel with plasma HIV RNA [7,8]. Several studies also reported declining HIV-specific memory CTL frequencies [9,10]. Rapid loss of activated T cells is appropriate with HIV antigen withdrawal, but concurrent disappearance of memory T cells suggests HIV-specific defects. In this context, a distinct distribution pattern of cell surface CCR7, CD27, and CD45RA reported for HIV versus cytomegalovirus (CMV)-specific CD8⁺ T cells indicates selective accumulation of immature HIV-specific memory cells [11-13]. Both antigen presenting cell and CD4⁺ helper T cell abnormalities could contribute to defective generation of HIV-specific CD8⁺ T cells, but more research is needed for a better understanding of factors affecting evolution of HIV-specific memory CTL during effective antiretroviral therapy [14,15].

To address this need, we monitored anti-HIV CTL activity and a number of potentially related factors in 44 HIV-infected individuals with different patterns of HIV replication. Individuals with advanced HIV infection, as previously defined by extremely low T cell counts, were excluded as anti-HIV CTL activity would be undetectable in part of this population at study onset [3,4]. The individuals included varied considerably with respect to extent of immune degeneration occurring before treatment, efficacy of their antiretroviral treatment and extent of immune reconstitution that followed treatment. Anti-HIV CTL activity persisted in all individuals with sustained detectable HIV replication and in most individuals with prolonged (>4 years) suppression of HIV replication to undetectable levels. However, about 1/3 of individuals with prolonged periods of undetectable plasma HIV RNA lost all anti-HIV CTL activity. To identify factors predisposing to this loss, we compared the group of individuals who lost HIV-specific memory CTL activity to those who retained their HIV-specific CTL over prolonged periods of effective antiretroviral therapy. This revealed immunological, virological and other factors associated with loss of HIV-specific CD8⁺ CTL following suppression of HIV replication to undetectable levels.

5.3 Materials and Methods

5.3.1 Study subjects

Study participants were recruited through the Infectious Disease Clinic of the St. John's General Hospital, St. John's, NL, Canada. Clinic visits were scheduled at approximately 3 month intervals or more often as necessary for appropriate clinical care. In conjunction with each visit clinical evaluation was performed, plasma HIV RNA was measured using Amplicor HIV-1 MonitorTM kits (Roche Diagnostic Systems Inc., Mississauga, Ontario, Canada) and peripheral blood lymphocyte (PBL) subsets were assessed by flow cytometry. Ethical approval was obtained from the Memorial University Faculty of Medicine Human Investigation Committee and all participants provided informed consent for blood collection and access to clinical and laboratory records.

5.3.2 Sample preparation

Peripheral blood mononuclear cells (PBMC) were isolated from acid-citrate dextrose (ACD)-preserved fresh whole blood by density gradient centrifugation with Ficoll-paque lymphocyte separation medium (Pharmacia Chemicals, Dorval, Quebec, Canada). PBMC were washed twice in phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS; Gibco, Grand Island, New York, USA), resuspended in lymphocyte medium (RPMI 1640 with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2×10^{-5} M 2-mercaptoethanol; all from Gibco) and counted. Human leukocyte histocompatibility antigen (HLA) class I typing and generation of B lymphoblastoid cell lines (BLCL) were done as previously described [4].

5.3.3 Cytotoxicity assays

To activate anti-HIV CTL, approximately 1×10^7 PBMC were stimulated by an autologous mixed lymphocyte reaction (AMLR) as previously described [4]. HIV-specific CTL activity was assessed against autologous or HLA-matched BLCL targets infected with the following recombinant vaccinia viruses: vDK1 (*gag*), vCF21 (*pol*), vPE16 (*gp160*), vTF*nef* (*nef*), and vSC8 (*E. Coli* β -galactosidase, control) (all from the NIH AIDS Research and Reference Reagent Program, Rockville, Maryland). Target cells were infected with 15 plaque forming units (pfu)/cell of the desired virus overnight, labeled for 90 minutes with 100 μ Ci $\text{Na}_2^{51}\text{CrO}_4$ (Amersham Life Sciences, Arlington, IL), washed 4 times with PBS containing 1% FBS, counted, and resuspended in lymphocyte medium. Effector and target cells (5000 cells/well) were incubated in duplicate in 96-well culture plates in a final volume of 300 μ l/well at effector:target (E:T) ratios of 50:1, 25:1, and 12.5:1. Minimum and maximum release wells contained targets in medium alone or 1N HCl respectively. After 5 hours incubation, 100 μ l supernatant was removed and counted in a Wallac 1480 gamma counter. Percent killing was determined by the formula:

$$\frac{(\text{test } ^{51}\text{Cr release} - \text{spontaneous release}) \times 100}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}$$

Spontaneous ^{51}Cr release was always <25% of maximum ^{51}Cr release and results were considered positive when % specific ^{51}Cr release in the test wells was $\geq 10\%$ above controls.

5.3.4 Flow Cytometry

To determine the proportion of CD8⁺ T cells expressing CD28, PBMC were washed in PBS with 5 mM EDTA and 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri, USA) and incubated for 20 minutes at 4⁰C with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (Dako Diagnostics, Mississauga, Ontario, Canada), phycoerythrin (PE)-conjugated anti-CD28 and Peridinin Chlorophyll Protein (PerCP)-conjugated anti-CD8 (Becton Dickinson, Mississauga, Ontario, Canada). Samples were then washed, resuspended in 0.5% paraformaldehyde in PBS and analyzed on a FACStarPlus flow cytometer (Becton Dickinson) after excitation at 488 nm.

5.3.5 Proliferation assays

Lymphocyte proliferation assays were carried out by resuspending PBMC in lymphocyte medium with 10% human AB serum (ICN Biomedicals Inc., Aurora, Ohio) replacing FBS. Cells were incubated at 1x10⁵/well in triplicate in 200 µl medium in 96-well culture plates. Negative control wells included 2 µg/ml β-galactosidase (VWR Canlab, Mississauga, ON) or medium alone, while positive control wells contained 5 µg/ml purified phytohemagglutinin (PHA-P; ICN Biomedicals Inc.). Test wells contained 2 µg/ml *Candida albicans* antigen (Greer Laboratories Inc., Lenoir, NC) or HIV p24 (Virogen, Watertown, MA). After 5 days, wells were pulsed with 1 µCi ³H-thymidine (Perkin Elmer Life Sciences Inc., Boston, MA), for 16 hours. The cells were then harvested onto filter mats using a Tomtec Harvester96 and counted on a Packard TopCount beta counter. Stimulation indices (SI) were determined by dividing ³H-

thymidine incorporation in test wells by ^3H -thymidine incorporation in negative control wells. An SI >5 was considered a positive response.

5.3.6 Statistical Analyses

Normal distribution of numerical data was confirmed by the Kolmogorov-Smirnov test. Significant differences in mean values of parameters compared between groups were assessed by one way ANOVA. Fisher's exact test was used to assess associations and linear regression was used to assess correlation between parameters. All univariate statistical analyses were done with SPSS for Windows, version 9 (SPSS Inc.) and had a power of at least 80%. A binary logistic analysis with both forward and backward conditional stepwise regression was used to evaluate variables independently associated with loss of HIV-specific CTL.

5.4 Results

5.4.1 Subject Characteristics

Forty-four HIV-infected individuals were included in this study. These individuals fell into 2 groups based on viral replication over the study period. Twenty had detectable plasma HIV RNA throughout (group 1). Of these individuals, 17 were receiving HAART, with 10 on protease inhibitor (PI)-based regimens and 7 on non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimens. Two received dual combination therapy with nucleoside analogue RT inhibitors (NRTI) and one subject was treatment naïve. In most cases, virus loads fluctuated around 1×10^4 copies/ml plasma during the study, but several individuals consistently maintained virus loads above 1×10^5 . Twenty-four subjects maintained undetectable plasma HIV RNA throughout (group 2). Eighteen of these individuals received PI-based HAART, 3 received combination therapy with 2 NRTIs and 4 received combination therapy with 3 NRTIs. Table 1 compares basic characteristics of the 2 groups. The group without detectable virus replication had significantly more CD4⁺ T cells at both early (average of first two clinic visits following study onset) ($p = 0.01$) and late (average of 2 most recent clinic visits) ($p = 0.004$) time points. However, these groups did not differ significantly with respect to gender, mean age, or mean CD8⁺ T cell counts at early or late time points (Table 1). All but the treatment naïve individual had received dual combination therapy with NRTIs prior to the introduction of NNRTIs and PIs.

Table 1. Comparison of general characteristics of groups with and without detectable HIV replication over the study period.

Parameter	Group 1 ^a (n = 20)		Group 2 (n = 24)	
	12 male, 8 female		14 male, 10 female	
	Range	Mean \pm SD	Range	Mean \pm SD
Age at study onset (years)	23 – 43	33.7 \pm 6.8	24 – 49	34.5 \pm 6.80
Follow-up (months)	14 – 49	32 \pm 12	16 – 56	37.0 \pm 10.8
CD4 ⁺ T cells – early ^b	30 – 812	345 \pm 202	192 – 898	475 \pm 184
CD4 ⁺ T cells – late	43 – 758	399 \pm 289	259 – 1474	618 \pm 215
CD8 ⁺ T cells – early	481 – 1353	965 \pm 544	254 – 2559	1015 \pm 314
CD8 ⁺ T cells – late	463 – 1575	1071 \pm 557	203 – 2454	968 \pm 328

^aGroup 1 is composed of individuals who had detectable HIV replication throughout the study. Group 2 is composed of individuals who maintained HIV replication at undetectable levels throughout the study.

^bAll T cell values are expressed as the number of cells/ μ l peripheral blood. Early refers to the mean of each individual's values measured at the first 2 time points in the study while late refers to the mean of the values measured at the last 2 study time points.

5.4.2 HIV-specific CTL Activity

Subjects were tested for CTL activity against HIV *gag*, *pol*, *env*, and *nef* over periods ranging from 14 – 56 months. At study onset, all had CTL activity against at least one HIV antigen. Figure 1a depicts the percentage of individuals within each group with CTL against each HIV antigen tested. HIV-specific CTL responses in groups 1 and 2 were similarly distributed with regard to overall specificity. All 20 individuals in group 1 with detectable levels of HIV replication maintained anti-HIV CTL activity throughout the study, compared to 17/24 subjects in group 2. Several of the 37 individuals maintaining anti-HIV CTL activity varied in terms of partial loss or broadening of activity, but responses against at least 1 HIV protein persisted throughout the study. HIV-specific CTL activity disappeared for 7 individuals in group 2 (group 2a). Loss of HIV-specific CTL activity in the total study cohort was strongly associated with suppression of viral replication below detectable levels ($p = 0.009$).

All 7 individuals who lost their anti-HIV CTL were receiving PI-based HAART. Figure 1b shows their pattern of HIV-specific CTL responses at baseline relative to the 17 individuals with persistent anti-HIV CTL activity despite viral suppression (group 2b). The mean number of HIV proteins (out of 4 tested) recognized by CTL was lower for group 2a than 2b ($p = .026$). When the mean amplitudes of individual CTL responses against *gag*, *pol*, *env* and *nef* were compared between groups 2a and 2b, the mean amplitude of anti-*gag* responses was significantly lower in group 2a ($p = .041$). Thus, there was evidence of weaker baseline anti-HIV CTL responses in the group of individuals who lost their responses during effective antiretroviral therapy.

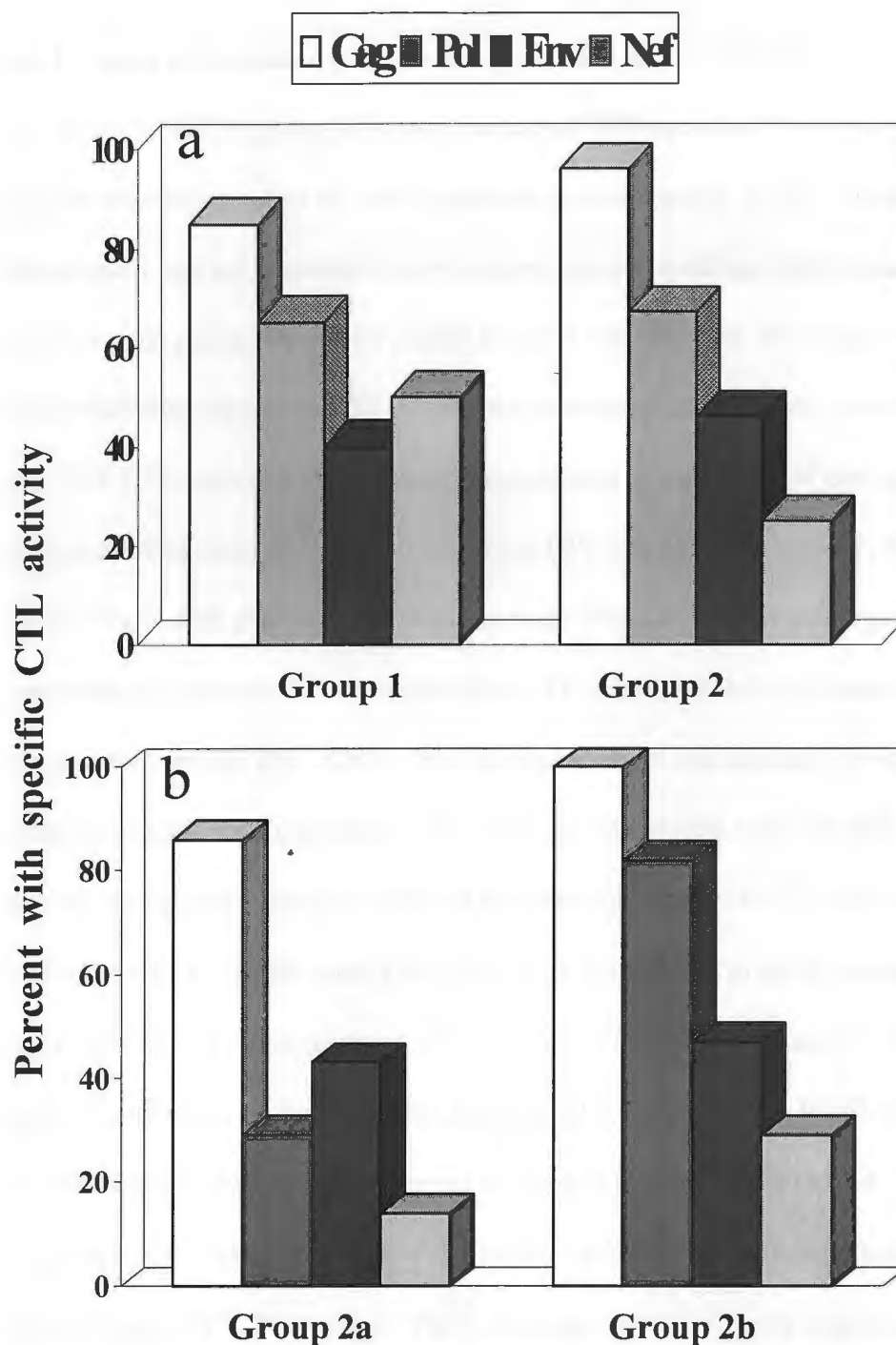


Figure 1. Comparison of anti-HIV CTL responses in different groups. Panel a compares the percentage of individuals with CTL against each of the indicated HIV antigens in groups with detectable (group 1) and undetectable (group 2) HIV replication throughout the study. Panel b shows the same comparison for those within group 2 who lost (group 2a) and those who maintained (group 2b) their HIV-specific CTL over the study period.

5.4.3 Factors Associated with Loss of Anti-HIV CTL Activity

For the 7 subjects in group 2a, complete loss of HIV-specific CTL occurred 18 to 50 months after suppression of viral replication to undetectable levels. Duration of viral suppression was not a critical factor as there was no significant difference in follow-up time between groups 2a and 2b (Table 2) and 5 subjects with the longest periods of controlled virus replication (51-56 months) maintained consistently strong and diverse anti-HIV CTL activity. To uncover factors related to the failure of certain individuals with controlled viral replication to maintain HIV-specific CTL activity, we compared the 7 individuals with prolonged viral suppression who lost CTL activity (group 2a) to the remaining 17 individuals who maintained CTL activity despite prolonged viral suppression (group 2b). Table 2 lists the mean, range and standard deviation (SD) of parameters used for comparison. The mean age of subjects who lost anti-HIV CTL activity was greater than that of the 17 who retained anti-HIV CTL activity ($p = .01$). Mean nadir $CD4^+$ T cell count (including time points prior to study onset, $p = .005$) and early $CD4^+$ T cell count (average of first 2 values after study onset, $p = .005$) were significantly lower in the group that lost anti-HIV CTL activity. While $CD8^+$ T cell counts were not significantly different for these 2 groups, the $CD4:CD8$ T cell ratio at the beginning ($p = .029$) and end ($p = .023$) of the study was significantly lower in the group that lost anti-HIV CTL activity. The percentage of $CD8^+$ T cells expressing CD28 also related to maintenance of anti-HIV CTL activity. Subjects who lost HIV-specific CTL activity had a lower mean percentage of $CD8^+$ T cells expressing CD28 at early and late time points ($p = .03$). Using past plasma virus load measurements, we also found that the group who lost anti-HIV CTL activity had experienced higher plasma HIV RNA levels

Table 2. Comparison of groups with and without sustained HIV-specific CTL responses over periods of undetectable HIV replication.

Treatment	Group 2a ^a	n = 7	Group 2b	n = 17	ANOVA	
	PI-based HAART	7	10			
	2 NRTIs	3				
	3 NRTIs	4				
Parameter	Range	Mean ± SD	Range	Mean ± SD	F	p
Age at study onset (years)	33-52	43 ± 7.35	28-50	36.4 ± 5.24	6.2	0.01
Duration viral suppression	23-45	37.7 ± 7.95	16-56	34.8 ± 12	0.34	0.29
Nadir CD4 ⁺ T cells ^b	18-314	160 ± 115	176-835	336 ± 152	7.5	0.005
CD4 ⁺ T cells - early ^c	172-487	310 ± 122	221-1046	551 ± 211	7.9	0.005
CD4 ⁺ T cells - late	259-757	483 ± 194	439-1131	660 ± 220	3.8	0.04
Nadir CD8 ⁺ T cells	417-1436	682 ± 356	163-1240	649 ± 308	0.05	0.41
CD8 ⁺ T cells - early	471-2844	1186 ± 764	254-3419	1052 ± 709	0.17	0.34
CD8 ⁺ T cells - late	582-2811	1101 ± 766	203-2795	985 ± 640	0.15	0.35
Peak virus load ^d	5-6	5.3 ± 0.6	3-6	4.25 ± 0.93	7.7	0.005
%CD8 ⁺ T cells CD28 ⁺ - early	27-44	42.4 ± 5.2	25-77	45.7 ± 15.0	4.03	0.03
%CD8 ⁺ T cells CD28 ⁺ - late	38-53	42.4 ± 5.2	33-71	51.1 ± 11.3	3.7	0.03

^aGroup 2a is composed of individuals with undetectable HIV levels throughout the study who lost their HIV-specific CTL activity. Group 2b is composed of individuals with sustained undetectable HIV replication throughout the study who maintained HIV-specific CTL activity.

^bAll T cell values are expressed as the number of cells/ μ l peripheral blood. The nadir value is the lowest observed historical value including prior to study onset.

^cEarly refers to the mean of each individual's values measured at the first 2 time points in the study while late refers to the mean of the values measured at the last 2 study time points.

^dThe peak virus load value is the highest observed historical value in \log_{10} copies of HIV RNA/ml plasma including prior to study onset.

before responding to therapy ($p = .005$). For the 7 individuals who lost HIV-specific CTL activity, there was a significant direct correlation between nadir $CD4^+$ T cell counts and duration of anti-HIV CTL persistence after viral replication was suppressed to undetectable levels ($r = .889$, $p = .007$; fig. 2a). There was a weaker inverse correlation with nadir $CD8^+$ T cell counts ($r = -.781$, $p = .038$; fig. 2b) and no significant correlation with any other parameter measured. Together, these factors outline a profile characterized by older age, more extensive previous disease progression and susceptibility to more rapid disease progression that reflects a limited capacity to maintain anti-HIV CTL activity in the absence of detectable replicating virus.

Multivariate analysis was carried out to evaluate which factors were independently associated with loss of anti-HIV CTL. Binary logistic regression demonstrated that early $CD4^+$ T cell counts ($p = .03$), anti-HIV CTL amplitude at study onset ($p = .04$) and the percentage of $CD8^+$ T cells expressing CD28 at late time points ($p = .03$) independently associated with loss of anti-HIV CTL (Table 3). The small sample size ($n = 7$) of the group who lost anti-HIV CTL limits the power of this test to exclude the factors not found to be independently associated with loss of anti-HIV CTL.

5.4.4 $CD4^+$ T Cell Function

To test whether the association between $CD4^+$ T cell numbers and persistent anti-HIV CTL activity reflected particular $CD4^+$ T cell functions, we measured T cell proliferation against PHA, Candida, and HIV p24 for 39 individuals. Figure 3a shows that all had proliferative responses to PHA with fewer responses against Candida and even fewer against HIV p24. Figure 3b shows the frequency of proliferative responses for group 2

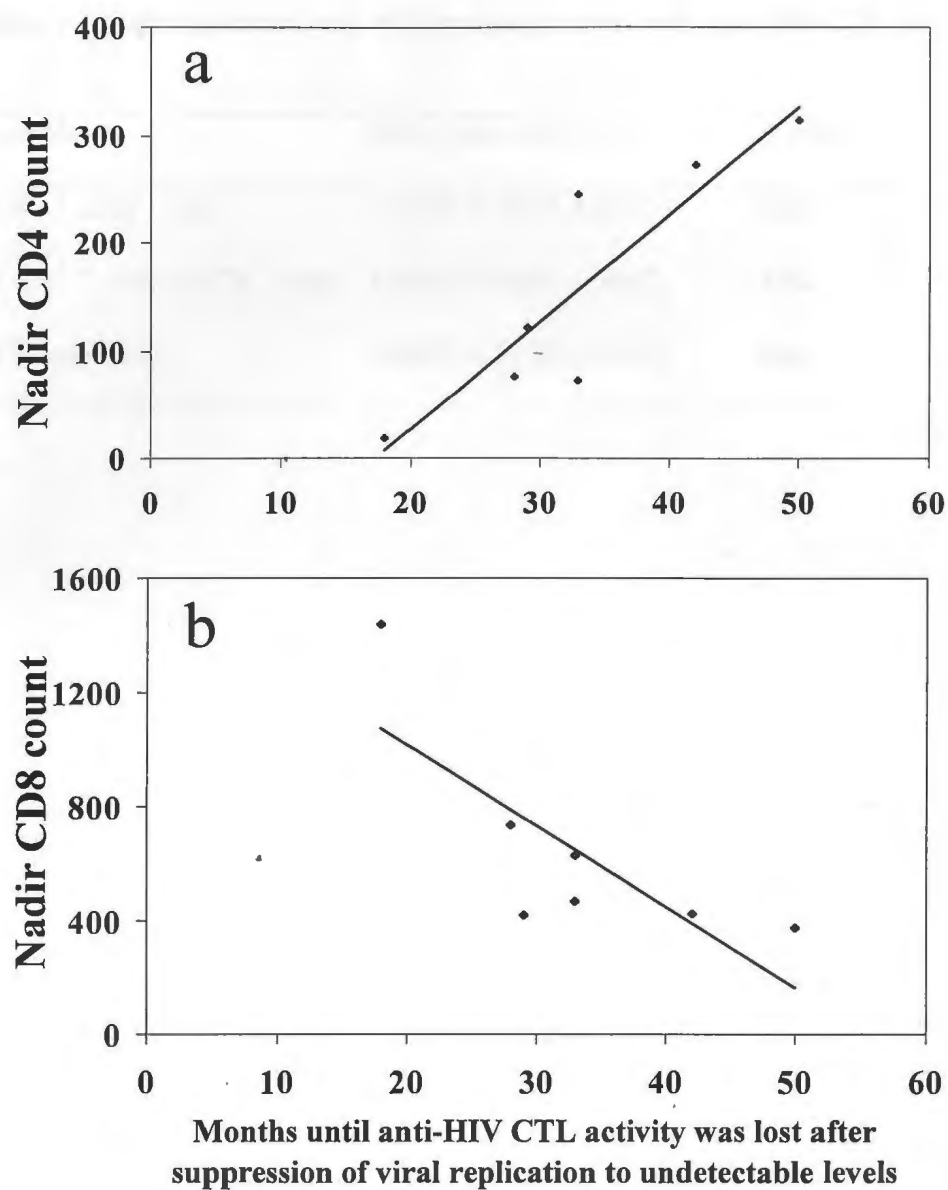


Figure 2. Relationship between nadir T cell counts, suppression of virus load to undetectable levels, and disappearance of HIV-specific CTL with proliferative potential. Scatter plots of the time HIV-specific CTL, following in vitro stimulation, persisted once HIV replication was suppressed below detectable levels, versus nadir CD4⁺ T cell counts (panel a) or nadir CD8⁺ T cell counts (panel b) are shown for the 7 individuals in group 2a who lost HIV-specific CTL activity. The line of best fit is shown in each case.

Table 3. Multivariate analysis of factors associated with anti-HIV CTL loss

Variable	Odds Ratio (95% CI)	p value
CD4 ⁺ T cells - early	1.0159 (1.0008-1.0313)	0.03
%CD8 ⁺ T cells CD28 ⁺ - late	1.1698 (1.0002-1.3682)	0.04
CTL amplitude	1.0920 (1.0152-1.1747)	0.03

(HIV replication suppressed to undetectable levels) expressed separately for the 7 individuals who lost CTL activity (group 2a) and for 14 of the individuals who maintained memory CTL activity (group 2b). Although it is noteworthy that none of the 7 individuals who lost CTL activity had proliferative responses to HIV p24, this was not significantly different from the frequency of p24-specific proliferation in any of the other groups. Therefore, we found no significant association between maintenance of HIV-specific CD8⁺ CTL responses and HIV-specific CD4⁺ T cell responses.

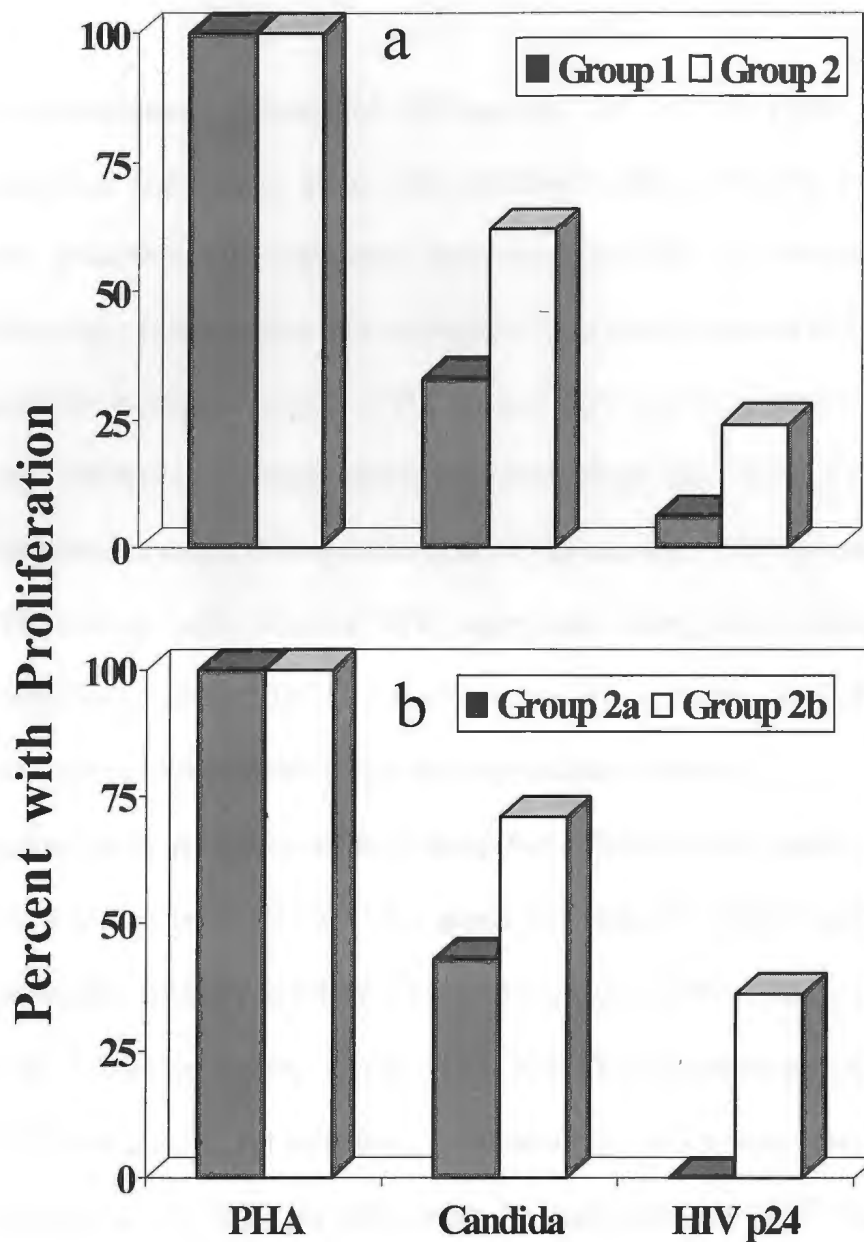


Figure 3. T cell proliferation responses in different groups. Figure 3a compares the percentage of individuals in groups 1 (detectable HIV replication) and 2 (HIV replication suppressed below detectable levels) with CD4⁺ T cell proliferation (SI >5) against PHA, Candida, and HIV p24. Figure 3b compares the same T cell responses in group 2 individuals who either lost (group 2a) or maintained (group 2b) HIV-specific CTL activity throughout the study.

5.5 Discussion

We investigated evolution of HIV-specific CTL in one group with persistent HIV replication and another group with sustained undetectable HIV replication. Everyone with detectable viral replication maintained anti-HIV CTL through up to 4 years of follow-up. This indicates that with chronic antigen stimulation HIV-infected individuals generally maintain anti-HIV CTL, at least until advanced infection. Most individuals with effective viral suppression also maintained anti-HIV CTL activity, indicating generation of stable HIV-specific CD8⁺ T cell memory. The minority who lost anti-HIV CTL activity with effective HIV suppression may bear a specific defect affecting generation of stable CD8⁺ T cell responses. It is important to understand the nature of this defect and any impact it may have on treatment options.

Factors we found associated with this defect reflected either duration of HIV infection or extent of disease progression. The group with defective CD8⁺ T cell memory were older, had weaker baseline anti-HIV CTL activity, fewer CD4⁺ T cells, a lower percentage of CD8⁺ T cells expressing CD28, lower nadir T cell counts and higher historical HIV replication levels. An increasing immunological deficit with more advanced disease is expected, but the lingering relationship between defective CD8⁺ T cell memory, nadir T lymphocyte counts and historical virus loads is more complex. There was a highly significant direct correlation between nadir CD4⁺ T cell count and time to HIV-specific CTL loss. This suggests that past disease progression, reflected by nadir CD4⁺ T cell counts, can have more relevance to certain immunological functions than current disease status, reflected by contemporary CD4⁺ T cell counts. Previous findings that lower nadir

CD4⁺ T cell counts are associated with persistent T cell proliferation defects, prolonged immune activation and weaker vaccine responses following HAART confirm lingering effects of past disease progression [16-18]. While HAART-related immune reconstitution restores protection against opportunistic pathogens common in advanced HIV infection, HIV-specific helper T cell responses are not restored with similar kinetics [19]. There may be a finite window period during which HIV replication must be effectively addressed to preserve HIV-specific T cell responses [20,21]. Selective depletion of HIV-specific CD4⁺ T cells is also consistent with an immunological burden of past disease progression beyond what current CD4⁺ T cell counts appear to indicate [22]. This may be an important consideration in basing the safety of treatment interruptions on return to baseline CD4⁺ T cell counts when substantial drops in CD4⁺ T cell count precede treatment resumption [23].

Several lines of evidence indicate that antigen-specific CD4⁺ helper T cells contribute to anti-HIV CTL responses [24,25]. Introducing HAART during acute infection preserves HIV-specific CD4⁺ T cell proliferation, which is associated with higher HIV-specific CTL frequencies [20]. In addition, individuals with limited HIV disease progression maintain strong HIV-specific CD4⁺ and CD8⁺ T cell responses [26]. Thus, early loss of HIV-specific CD4⁺ T cells may be an important determinant of disease progression and HIV-specific CTL loss during effective antiretroviral therapy may reflect previous loss of HIV-specific helper T cells. In our study, individuals who lost HIV-specific CTL responses were uniformly negative for HIV-specific T cell proliferation. However, only 2/20 individuals with chronic HIV replication who retained HIV-specific CD8⁺ T cell responses had detectable HIV-specific CD4⁺ T helper cells. This supports the suggestion

of Wodarz et al. that CTL responses persist without antigen while helper T cells are present, but require high levels of antigen to persist without helper cells [27]. Persistence of anti-HIV CTL with detectable viral replication in the absence of HIV-specific helper T cells may involve continuous generation of effector cells without replenishing the memory cell pool. Continuous recruitment of memory and naïve T cells into the terminally differentiated effector population with limited proliferative potential is consistent with the higher proportions of CD28⁻ CD8⁺ T cells in individuals with chronic HIV replication [28,29].

The stability of HIV-specific CTL responses may impact on any potential utility of treatment interruption in several ways. Firstly, individuals who lose HIV-specific CTL activity on HAART should suffer more rapid and deleterious changes with treatment interruption. Even those HIV-infected individuals retaining anti-HIV CTL activity respond less vigorously to viral rebound than to primary infection [30]. Nonetheless, a rise and fall of HIV levels following treatment interruption suggests useful reengagement of the immune system, presumably favoured in individuals retaining anti-HIV CTL [31]. Secondly, the usefulness of stimulating unstable immunity against HIV by treatment interruption would be limited. Individuals who lose anti-HIV CTL activity on HAART appear less likely to generate stable CTL responses with treatment interruption and less likely to benefit. Therefore, robust anti-HIV CTL responses may identify individuals most likely to benefit from treatment interruptions. Conversely, weakening anti-HIV CTL activity might identify individuals requiring immunological conditioning to benefit from treatment interruption. More studies comparing individuals with stable versus transient anti-HIV CTL activity are needed to clarify the role of HIV-specific CD4⁺ T

cells, antigen-presenting cell status and other factors in generating stable CD8⁺ T cell responses.

In summary, there is a clear discrepancy in some HIV-infected individuals between the ability to generate anti-HIV CTL and the ability to maintain them over prolonged periods of undetectable viral replication. The inability to maintain anti-HIV CTL is associated with historical or current progression to more advanced disease. Therefore, in some respects historical disease progression may be equally as relevant to anti-HIV immune status as current T lymphocyte counts.

5.6 Acknowledgements

The authors would like to thank the United States National Institute of Allergy and Infectious Diseases (NIAID) AIDS Reference Reagent Program for providing recombinant vaccinia viruses. We also thank the HIV-infected individuals who participated in this study.

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CHAPTER 6

General Summary and Discussion

6.1 Preface

The work described in this thesis sought to further elucidate the biological relevance of anti-HIV CTL. Defining the precise role of antigen-specific CTL in HIV infection has proven problematic. While it is now generally accepted that anti-HIV CTL play a key role in controlling disease progression, it is also clear that multiple mechanisms contribute to their failure to indefinitely control virus replication. The experiments conducted in this thesis addressed possible mechanisms responsible for the ultimate failure.

The thesis basically involved two major studies. In the first, we performed a detailed examination of the CD8⁺ T cell population persisting through the advanced stages of HIV infection. This study yielded intriguing results regarding the longevity and CD28 phenotype of HIV-specific memory CTL. For individuals with advanced HIV infection, anti-HIV memory CTL were predominantly CD28⁻, and were detectable only when replicating virus was detectable. To address these issues further, the remainder of the project focused on examining HIV-specific memory CTL responses within a large group of chronically infected individuals at various stages of disease.

Most of the experimental details and results have been summarized in the four manuscripts presented as chapters 2-5. In the ensuing sections of this chapter the overall significance of the information gained through these studies will be discussed.

6.2 CD8⁺ T Cells in Advanced HIV Infection

When this study was initiated it was uncertain whether normal functional attributes of CD8⁺ T cells persisted through the advanced stages of HIV infection. It was known that circulating HIV-specific CTL activity declined in parallel with disease progression, but it was unclear whether the same was true for anti-HIV CTL precursors (1). We examined CD8⁺ T cells from individuals with advanced HIV infection in terms of functional ability, HIV-specific CTL activity, and TCR β V repertoire diversity. Our data clearly showed that CD8⁺ T cells from advanced stage individuals proliferated and differentiated into functional CTL *in vitro*, and that TCR β V diversity was maintained. In addition, for more than half the subjects we studied, anti-HIV precursor CTL activity was not only detectable but, in some cases, was strong and diverse.

We found circulating CTL activity to be completely absent in the group of individuals with advanced HIV infection, whereas it was common to all subjects at less advanced stages of disease. Although we expected HIV-specific circulating CTL activity to be undetectable, the complete absence of circulating, effector CTL was unexpected. The complete lack of effector CTL activity suggested that an HIV-induced, *in vivo* defect in the ability to generate an effector CTL response arises during progressive disease. The

precursor cells themselves were not intrinsically defective, as shown by their *in vitro* activation and differentiation into functional CTL. Therefore, the defect must lie elsewhere. In our opinion, low total CD4⁺ T cell help was the most likely explanation. At late stages of infection, CD4⁺ T cells are present in extraordinarily low numbers. Indeed, several individuals in our study had no detectable peripheral blood CD4⁺ T cells. Given the paucity of CD4⁺ help at this late stage of infection, we hypothesized that CD8⁺ T cells, though functional, were not receiving adequate activation signals for proliferation and differentiation. Over the course of our study, four subjects had their antiretroviral therapy regimes modified, which resulted in control of viral replication and increasing T cell numbers. For these subjects, circulating CTL activity did become apparent, indicating that the *in vivo* defect in the ability to generate activated CTL can be overcome even after sustained periods of advanced infection. Surprisingly, for two individuals who initially showed a lack of HIV-specific precursor CTL activity, this activity also became apparent once effective therapy was initiated. This demonstrated that the HIV-specific CTL response was not exhausted for these subjects, as had originally been suspected.

The findings of this study indicated that the CTL response to HIV, and presumably to other antigens as well, is remarkably durable. Even under conditions whereby the immune system is exposed to persistent antigenic stimulation for extended periods, with deterioration of various immune components, CTL precursors persist and retain their normal functional attributes. In extreme circumstances, when the immune system has suffered severe deterioration, CTL precursors may be precluded from differentiating into effector CTL and performing their protective lytic function. However, this condition is reversible. If circumstances improve, precursors can again become activated to proliferate

and differentiate into circulating, effector CTL. Therefore, CTL based immunotherapy for HIV and other pathogens is a plausible consideration even for those individuals who have progressed to an advanced stage of disease.

It is clear, however, that anti-HIV CTL do not afford lifelong protection from progression to AIDS. Disease progression can occur in the face of strong and diverse HIV-specific CTL responses (2). There were two other findings in our study of advanced stage individuals that we felt could shed light on reasons for the ultimate demise of the anti-HIV CTL response. The first was the finding that, although peripheral circulating T cells were predominantly CD28⁺ for the advanced stage subjects we tested, anti-HIV precursor CTL were predominantly CD28⁻, suggesting that loss of CD28 expression on CTL might be associated with disease progression. The second finding was the fact that anti-HIV CTL responses for the four individuals who responded to therapy were transient, detectable only when virus was replicating and disappearing rapidly once replication was brought under control. This finding indicated an inability to maintain memory CTL when antigen supply was limited, which conceivably would contribute to disease progression.

We wanted to determine whether these two features were peculiar to HIV-specific CTL at the advanced stages of infection, or common to other phases of disease. If either (or both) attribute was predominant during advanced infection only, this might indicate a particular characteristic of anti-HIV CTL that occurs concurrent with deteriorating disease, and results in acceleration of disease progression. The remainder of this thesis project focused on examining these two attributes within a larger group of HIV infected individuals who were at various stages of infection.

6.3 A comparison of CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells

We compared CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells within a group of 41 chronically infected individuals. The individuals varied considerably with respect to concurrent disease activity as well as cumulative disease progression, and thus, represented a broad cross-section of HIV disease status.

In the first part of the study the CD28 phenotype of HIV-specific CTL was determined. We found a strong correlation between accumulation of CD28⁻ anti-HIV CTL and markers of disease severity. When CD28⁻ CTL were prevalent, viral load levels were high and CD4⁺ T cell counts were low. Conversely, for individuals demonstrating suppression of viral replication and higher CD4⁺ T cell counts, anti-HIV CTL usually included CD28⁺ T cells. This concurred with our results for subjects with advanced infection, reinforcing the suggestion that accumulation of CD28⁻ HIV-specific CTL is associated with disease progression.

We then compared CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells in terms of TCRβV repertoire diversity and cytokine production. We hypothesized that if disease progression was directly related to accumulation of CD28⁻ CTL, this might be reflected by an altered βV repertoire and/or lower cytokine production output by the CD28 negative population. We also proposed that individuals with more advanced disease would have greater perturbations in their CD28⁻ T cell subset than subjects with more limited disease progression.

Although we did find CD28⁻ T cells to have a less diverse TCRβV repertoire than their CD28⁺ counterparts, this was true for all individuals tested, regardless of disease stage. CD28 negative T cells produced both IL-2 and IFN-γ, and the number of cells producing these cytokines was similar for all individuals tested. Therefore, there was no apparent deterioration of the CD28⁻ T cell population associated with disease progression.

In HIV infection, CD28⁻ T cells expand during primary infection and often comprise more than 60% of the peripheral blood CD8⁺ T cell population throughout the natural course of disease (3). This predominance of CD28⁻ T cells reflects continuous differentiation of CD8⁺CD28⁺ T cells into CD28⁻ effector cells in response to chronic virus stimulation. The proportion of CD8⁺ T cells expressing CD28 also decreases with age, indicating that prolonged antigenic experience eventually leads to loss of CD28 expression even on resting memory cells (4). These CD8⁺CD28⁻ T cells have shortened telomeres that signify a long history of cell division culminating in replicative senescence, defined as an irreversible state of growth arrest that occurs when a cell has undergone its finite and predictable number of cell divisions (5). Therefore, CD28⁻ T cells do not represent a uniform population. Some are effector cells actively engaged in cell-mediated immune responses, while others are functionally effete remnants of past immunological battles. In our experiments, individuals with asymptomatic HIV infection usually demonstrated both CD28⁺ and CD28⁻ HIV-specific CTL. The CD28⁻ CTL were likely comprised mainly of effector cells, continuously being generated from their CD28⁺ counterparts. Individuals with progressive or advanced disease were much more likely to have anti-HIV CTL that were predominantly or exclusively CD28⁻. In these cases, we

propose that the detected CD28⁻ CTL were mainly cells in a state of replicative senescence. This supposition is based on 2 previous findings. First, without corresponding CD28⁺ HIV-specific CTL, it is unlikely that new effector cells were being generated, as studies have clearly demonstrated that CD28⁺ T cells generate CD28⁻ progeny (6). Second, we detected an *in vivo* defect in the ability to generate effector CTL in individuals with advanced HIV infection. It is, therefore, highly unlikely that the detected CD28⁻ CTL for these individuals represented new effectors, but rather a senescent population persisting after a long battle with HIV.

In summary, we found a clear relationship between HIV disease progression and the proportion of HIV-specific CTL expressing CD28. It appears that when uncontrolled virus replication occurs, HIV-specific CTL responses eventually become spent in their ability to recruit new effector cells from the CD28⁺ T cell population. As a result, CD28⁻ CTL accumulate with progression to AIDS.

6.4 HIV-specific Memory CTL Responses

We performed a longitudinal study of anti-HIV memory CTL responses with a group of 44 chronically infected individuals at various stages of disease. The individuals varied considerably with respect to the extent of immune degeneration that had occurred prior to HAART and the extent of immune reconstitution occurring subsequent to HAART. We observed persistent anti-HIV CTL activity in all individuals with sustained detectable plasma HIV RNA, and in the majority of those who responded to HAART with prolonged suppression of HIV replication to undetectable levels. None of the subjects

showed the same pattern of anti-HIV memory CTL that we observed for advanced stage individuals (who were excluded from this study), ie, rapid disappearance of HIV-specific CTL immediately following suppression of viral replication to undetectable levels. However, for 1/3 of the chronically infected individuals who experienced prolonged periods of undetectable plasma HIV RNA, HIV-specific CTL activity completely disappeared over the course of the study period. This loss occurred between 18 and 50 months following suppression of viral replication to undetectable levels.

When we compared various immunological and virological characteristics of individuals who lost HIV-specific CTL activity to those who retained their anti-HIV CTL over prolonged periods of effective treatment, we found that an individual's past history of disease progression was the most relevant factor related to their inability to maintain stable HIV-specific memory CTL responses. Nadir $CD4^+$ T lymphocyte numbers had the most striking relationship to defective $CD8^+$ T cell memory and there was a highly significant direct correlation between nadir $CD4^+$ T cell counts and the duration that HIV-specific CTL activity remained detectable. When these results were examined in the context of our earlier results for advanced stage individuals, a pattern emerged. Subjects who experience periods of uncontrolled virus replication resulting in significant depletion of $CD4^+$ T cells are likely to show evidence of defective $CD8^+$ memory T cell maintenance, with the severity of the defect proportional to the extent of $CD4^+$ T cell depletion. In our study, individuals who had progressed to advanced infection with extremely low $CD4^+$ T cell counts demonstrated a complete inability to maintain anti-HIV memory cells in the absence of detectable virus. Those who had suffered significant but less extensive depletion of their $CD4^+$ T cell population were able to maintain $CD8^+$

memory cells for a certain period of time, while stable HIV-specific memory CTL were maintained in the absence of detectable virus only for those individuals who had not sustained severe CD4⁺ T cell depletion. This data is consistent with recent evidence indicating that, in HIV infection, low nadir CD4⁺ T cell counts have prolonged effects on other aspects of T cell function. Persistent proliferation defects, increased T cell activation, and poor responses to immunization have been linked to past periods of CD4⁺ T cell lymphopenia (7,8,9). This suggests that long term exposure to HIV causes damage to the immune system that is difficult to correct. Delaying initiation of HAART may therefore result in impaired functional immune restoration despite normalization of circulating CD4⁺ T cell numbers.

These results indicate that the long-term quality of HIV-specific memory CTL responses may be markedly influenced by the specific nature of an individual's disease course, with a highly aggressive infection resulting in memory cells that can no longer persist in the absence of antigen. Significant HIV induced CD4⁺ T cell depletion at any time during the course of infection may have a negative impact on subsequent maintenance of an effective HIV-specific memory CTL response. This concurs with accumulating evidence indicating that HIV-specific CD4⁺ helper T cells contribute substantially to anti-HIV memory CTL responses (10,11). A recent study by Janssen et al confirmed the importance of antigen-specific helper cells in creating long-term memory CTL (12). This *in vivo* study utilized CD4-intact and CD4-deficient mice. Both sets of mice generated a primary response to antigen, with differentiation of CD8⁺ T cells into cytotoxic effectors. However, secondary CTL expansion was wholly dependent on the presence of helper T cells during priming. This indicates that CD4⁺ T cells are dispensable for primary

responses to antigen, but are absolutely required for the generation of long-term memory CTL.

To determine if our data on HIV-specific memory CTL activity in humans supported the interpretation of Janssen's study, we examined T cell proliferation responses for the individuals included in our study. For subjects who lost their anti-HIV CTL when treated successfully with HAART, none demonstrated proliferation to HIV p24. This appears to support the notion that lack of antigen-specific helper T cells is associated with impaired long term memory CTL responses. When we examined T cell proliferation responses for individuals treated successfully with HAART who maintained anti-HIV memory CTL, slightly less than half showed proliferation to HIV-specific antigen. At first glance, this appeared to conflict with Janssen's study, in that stable memory CTL responses were apparent and, therefore, antigen-specific helper T cells should be detectable. However, Janssen's study clearly demonstrated that secondary CTL expansion was dependent on the presence of T cell help during, but not after, priming. Since we tested for proliferation long after priming had occurred, it is entirely possible that HIV-specific CD4⁺ T cells were present during priming, but were subsequently lost. We propose that this group of individuals maintained HIV-specific helper T cell responses during the priming period, conferring on their CD8⁺ T cells the capacity for long term immunological memory.

Recent studies have focused on investigating the mechanisms by which T cell memory is generated and maintained. Subsets of memory T cells have been identified based on expression of cell surface markers, particularly CCR7 (13). Functional differences were noted for these memory T cell subsets as well. Based on these findings, two functionally

distinct memory T cell subsets were proposed: CCR7⁻ effector memory cells and CCR7⁺ central memory cells. When these subsets were examined in HIV- and CMV-infected individuals two effector memory cell subsets were distinguishable based on CD45RA expression (14). The HIV-specific cell pool was predominantly composed of pre-terminally differentiated CD8⁺CD45RA⁻CCR7⁻ cells, whereas the CMV-specific pool consisted mainly of terminally differentiated CD8⁺CD45RA⁺CCR7⁻ cells. High levels of HIV replication together with lack of adequate HIV-specific CD4⁺ T cell help were proposed to keep HIV-specific memory CD8⁺ T lymphocytes at the immature stage of CD8⁺CD45RA⁻CCR7⁻ cells, preventing their further differentiation. This skewed maturation of HIV-specific memory CD8⁺ T cells during HIV infection may have functional consequences for HIV pathogenesis. Indeed, in a recent study involving structured treatment interruption, an accumulation of pre-terminally differentiated (CD45RA⁻CCR7⁻) CD8⁺ T cells was associated with viral rebounds, demonstrating that failure to control viremia was related to accumulation of CD8⁺ T cells with an immature phenotype (15).

Determining the memory phenotype of anti-HIV CTL for individuals with no detectable antigen-specific helper T cell responses might provide a method of distinguishing between those who lose their HIV-specific CD4⁺ T cells early in the course of infection, resulting in defective memory CTL, and those who maintain HIV-specific CD4⁺ T cells beyond the priming period, creating an efficient memory CTL pool. This distinction could be useful when considering therapeutic regimes. For example, individuals who lose HIV-specific CD4⁺ T cells early during the course of infection, creating defective memory CTL maintenance, are more likely to experience deleterious consequences from

viral breakthroughs during HAART treatment, and would likely benefit from some form of immunological conditioning concurrent with their antiretroviral regimens.

The current study was completed before the results discussed above were available. This is unfortunate, as it would have been interesting to phenotype the memory cells of the subjects studied here, to determine if a relationship exists between memory cell phenotype and persistence. It is entirely possible that individuals demonstrating loss of CTL activity have high numbers of non-functional, pre-terminally differentiated CD8⁺ T cells.

In summary, our results demonstrate that individuals with detectable viral replication maintain anti-HIV CTL activity, at least until the very advanced stages of infection. However, this may reflect continuous generation of effector cells in response to chronic antigenic stimulation, without concomitant preservation of stable memory cells. The majority of individuals with efficient viral suppression also maintain anti-HIV CTL activity, suggesting that most HIV-infected subjects generate stable HIV-specific memory T cells. A minority of individuals lose their HIV-specific CTL activity during periods of efficient suppression of viral replication, and this may indicate a specific defect in the ability to maintain stable memory CTL responses. Our results suggest that an individual's past history of disease progression, particularly as indicated by nadir CD4⁺ T cell count, may have an important impact on the longevity of their CD8⁺ memory cells.

6.5 Concluding remarks

The fact that HIV disease progression can occur despite strong anti-HIV CTL responses undermines the importance of virus-specific CTL in controlling HIV infection. Ultimate failure of CD8⁺ T cell function should not be surprising given certain well understood characteristics of HIV pathogenesis. HIV can establish latent cellular infection, with provirus integrating directly into the host genome without concurrent expression of viral proteins. Thus, a persistent viral reservoir, undetectable to the immune system, is created. Virus and host co-exist for extended periods, with cumulative deleterious effects imposed upon various immune components. Anti-HIV CTL, although remarkable in terms of their diversity and magnitude, are low in frequency compared to the total number of HIV-infected cells replicating virus. The rapid rate of HIV replication, combined with the high error rate of reverse transcriptase, results in the generation of an extraordinary number of mutated virions, some of which escape CTL recognition (16,17). When these factors are taken into account, the ultimate failure of HIV-specific CTL responses is not surprising. It also becomes clear that multiple mechanisms could conceivably contribute to this ultimate failure.

The results obtained from this thesis project highlight some of the mechanisms involved in the progressive deterioration of anti-HIV CTL responses: an *in vivo* defect in the ability to generate an effector CTL response becomes evident during the late stages of disease; CD28⁻ HIV-specific CTL accumulate with chronic antigen stimulation; a defect in the ability to maintain stable anti-HIV memory T cells in the absence of detectable virus load occurs subsequent to severe CD4⁺ T cell depletion. All three mechanisms have

potential for weakening HIV-specific CTL responses. However, numerous other properties of HIV-specific CTL that might explain their inadequate immunoprotective role in HIV infection have also been identified (reviewed in chapter one). More focused research is required to understand the relative contribution of these diverse and sometimes unique mechanisms to CD8⁺ T cell dysfunction in HIV disease. Ideally this information will accelerate the development of effective vaccines and immunotherapeutic intervention strategies.

6.6 References

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